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AUTONOMIC CHOLINERGIC NEUROTRANSMISSION IN THE RESPIRATORY SYSTEM: EFFECT OF ORGANOPHOSPHATE POISONING AND ITS TREATMENT

A THESIS SUBMITTED TO THE UNIVERSITY OF OSLO FOR THE DOCTOR SCIENTIARUM DEGREE

BY

PER WALDAY

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The present study has shown that the organophosphate anticholinesterase soman inhibits the acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and carboxylesterase (CarbE) activities in the respiratory system in vitro (rat and guinea pig) and in vivo after inhalation exposure (rat). The rat and guinea pig in vitro bronchial smooth muscle response and the rat ex vivo tracheal response to nerve-mediated stimulation, were substantially inhibited by soman. This was probably due to an increased basal tone of the smooth muscle, induced by decreased inactivation and subsequent accumulation of acetylcholine (ACh) in the neuroeffector junctions. In vitro bronchial contractions induced by histamine in guinea pig were, however, not affected by soman, indicating that soman does not affect the airway smooth muscle itself but only the cholinergic system. During inhalation with soman, CarbE both in the respiratory system and plasma may function as a detoxifying scavenger.
Addition of HI-6 and Toxogonin after in vitro exposure to soman reestablished the rat tracheal smooth muscle contraction when added 10 min, but not 30 min, after soman. The recovery induced

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ABSTRACT (continued)

by Toxogonin was, however, not stable and not significantly different from control 90 min after addition of Toxogonin. HI-6 consistently produced a better recovery of the rat cholinergic ex vivo response than did Toxogonin, both alone and in combination with pyridostigmine pre-treatment. These effects could not easily be explained solely by AChE reactivation, since the ChE activities were not significantly different in treated and untreated animals. The importance of the time lapse between soman and HI-6 in vitro for the effectiveness of HI-6 suggest, however, that enzyme reactivation is the mechanism.

The organic calcium antagonists gallopamil, verapamil and diltiazem inhibited soman and nerve-mediated rat bronchial smooth muscle contractions in vitro, although only at high concentrations (μM). The mechanism behind the effect of these antagonists were probably dominantly inhibition of the calcium channels in smooth muscle. The peptide calcium antagonist, ω-conotoxin GVIA, effectively inhibited the contractions induced by stimulation of cholinergic nerves, but was ineffective against carbachol induced contractions, supporting the assumption that ω-conotoxin GVIA inhibits the calcium flux through channels responsible for the triggering of neurotransmitter release.

The rat bronchial smooth muscle contractions induced by stimulation of cholinergic nerves were enhanced by a methylxanthine sensitive mechanism after addition of purine nucleosides. This effect was probably mediated by stimulation of prejunctional adenosine receptors, since the adenosine analogue NECA enhanced the potassium induced release from bronchial smooth muscle in a methylxanthine sensitive manner.

PREFACE

The work presented in this thesis was carried out at the Norwegian Defence Research Establishment, Division for Environmental Toxicology, in the period 1988–1991. It is a part of the research program "The toxic effect of soman on the respiratory system" which was completed in 1991.

I am indebted to dr Pål Aas and professor Frode Fonnum for their excellent supervision, stimulating support, and constructive criticism.

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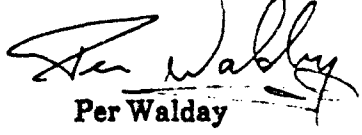

Per Walday

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ORIGINAL PAPERS

AUTONOMIC CHOLINERGIC NEUROTRANSMISSION IN THE RESPIRATORY SYSTEM: EFFECT OF ORGANOPHOSPHATE POISONING AND ITS TREATMENT

A thesis submitted to the University of Oslo for the Doctor Scientiarum degree

SUMMARY

The present study has shown that the organophosphate anticholinesterase soman inhibits the acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and carboxylesterase (CarbE) activities in the respiratory system *in vitro* (rat and guinea pig) and *in vivo* after inhalation exposure (rat). The rat and guinea pig *in vitro* bronchial smooth muscle response, and the rat *ex vivo* tracheal response to nerve-mediated stimulation, were substantially inhibited by soman. This was probably due to an increased basal tone of the smooth muscle, induced by decreased inactivation and subsequent accumulation of acetylcholine (ACh) in the neuroeffector junctions. *In vitro* bronchial contractions induced by histamine in guinea pig were, however, not affected by soman, indicating that soman does not affect the airway smooth muscle itself but only the cholinergic system. During inhalation with soman, CarbE both in the respiratory system and plasma may function as a detoxifying scavenger.

Addition of HI-6 and Toxogonin after *in vitro* exposure to soman reestablished the rat tracheal smooth muscle contraction when added 10 min, but not 30 min, after soman. The recovery induced by Toxogonin was, however, not stable and not significantly different from control 90 min after addition of Toxogonin. HI-6 consistently produced a better recovery of the rat cholinergic *ex vivo* response than did Toxogonin, both alone and in combination with pyridostigmine pretreatment. These effects could not easily be explained solely by AChE reactivation, since the ChE activities were not significantly different in treated and untreated animals. The importance of the time lapse between soman and HI-6 *in vitro* for the effectiveness of HI-6 suggest, however, that enzyme reactivation is the mechanism.

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The rat bronchial smooth muscle contractions induced by stimulation of cholinergic nerves were enhanced by a methylxanthine sensitive mechanism after addition of purine nucleosides. This effect was probably mediated by stimulation of prejunctional adenosine receptors, since the adenosine analogue NECA enhanced the potassium induced release from bronchial smooth muscle in a methylxanthine sensitive manner.

1 AIM OF THE STUDY

The present investigation was carried out to study different aspects of cholinergic autonomic neurotransmission in relation to the therapy of organophosphorus poisoning. The autonomic cholinergic neurotransmission in tracheal and bronchial smooth muscle in rat and guinea pig have been used as models. The following aspects have been studied: (1) effects of organophosphorus intoxication and treatment by carbamates, oximes, and calcium antagonists on the response of the airway smooth muscle *in vitro* and *ex vivo*. (Paper I, II, and III), (2) the role of carboxylesterase in the respiratory system during inhalation of organophosphorus compounds (Paper IV), and (3) the modulatory effect of adenosine and the effect of methylxanthines on the rat airway cholinergic neurotransmission (Paper V).

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

Paper I Aas P, Walday P, Tansø R, Fonnum F (1988): The effect of acetylcholinesterase inhibition on the tonus of guinea pig bronchial smooth muscle, *Biochem Pharmacol* 37, 4211–6.

Paper II Walday P, Aas P, Fonnum F (1992): The effect of pyridostigmine pretreatment, HI-6 and Toxogonin treatment on the rat tracheal smooth muscle response after organophosphorus inhalation exposure, *Arch Toxicol* (In press).

Paper III Walday P, Fyllingen E, Aas P (1992): Effects of calcium antagonists (ω-conotoxin, verapamil, gallopamil, diltiazem) on bronchial smooth muscle contractions induced by soman, *Naunyn-Schmiedeberg's Arch Pharmacol* (In press).

Paper IV Walday P, Aas P, Fonnum F (1991): Inhibition of serine esterases in different rat tissues following inhalation of soman, *Biochem Pharmacol* 41, 151–3.

Paper V Walday P, Aas P (1991): Prejunctional stimulation of cholinergic nerves in rat airway smooth muscle by an adenosine analogue, *Pulm Pharmacol* 4, 114–9.

2 GENERAL INTRODUCTION

2.1 Organophosphorus poisoning

Knowledge of the intoxication mechanism by organophosphorus compounds is important, since they are extensively used as insecticides in agriculture and some are potential warfare agents. The main uptake routes are by inhalation (during processing, manufacturing and application of insecticides, or by exposure to nerve agents on the battle field), the oral route (suicide attempts), or percutaneously. Organophosphorus compounds are rapidly distributed throughout the body regardless of route of uptake, and some insecticides have been shown to persist in the body for several days after intoxication (Braeckman et al 1983).

The acute toxic effect of organophosphates are due to inhibition of the enzyme acetylcholinesterase (AChE). This enzyme is responsible for breakdown and thereby inactivation of acetylcholine (ACh), which is the main neurotransmitter in cholinergic nerves. Inhibition of AChE results in accumulation of ACh in the synaptic junctions, and consequently excessive stimulation of the cholinergic receptors. Cholinergic synapses are present in both the peripheral and the central nervous system. The symptoms of intoxication at low doses are mainly due to inhibition of AChE in the autonomic nervous system, and includes miosis, hypersalivation, dyspnea, increased gastrointestinal motility, and bradycardia. Higher doses affect the myoneural plate and produce muscular symptoms ranging from fasciculations and weakness, to total paralysis. Still higher doses produce effects also in the central nervous system (CNS), such as convulsions and coma.

Much attention has been given to 1,1,2-trimethylpropyl methylphosphonofluoridate (soman), a highly toxic nerve agent (Figure 2.1). Soman has two asymmetric centres, and exists therefore as four different isoforms (C(–)P(+), C(–)P(–), C(+)P(+), and C(+)P(–); C stands for the asymmetric carbon and P for the asymmetry around the phosphorus atom). The P(–) isomers have a substantially higher affinity towards AChE, and are consequently much more toxic than the P(+) isomers (Benschop 1975). Toxicokinetic studies have revealed a two to three compartment behaviour in rat, guinea pig, dog, and marmoset (Benschop et al 1987; De Bisschop et al 1987). The elimination process occurs mainly in the liver and has been shown to be stereoselective, with a much more rapid hydrolysis of

the nontoxic isomers P(+) than the toxic isomers P(-) (Benschop et al 1981; Nordgren et al 1984).

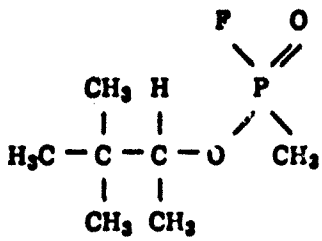

Figure 2.1 Molecular structure of soman

Current therapy of acute organophosphorus poisoning depends on the severity of the poisoning, but generally includes an antimuscarinic agent (atropine or scopolamine) plus an oxime (Lotti 1991). Oximes reactivate the inhibited AChE, and the reaction produces phosphylated oxime which is usually unstable and rapidly broken down to oxime and alkylphosphonic acid (Wilson and Ginsburg 1955; Fonnum 1975). The combination of an antimuscarinic agent and an oxime is an effective treatment against intoxication of almost all organophosphates, but most oximes have been shown to be without effect after intoxication with the nerve agent soman (Heilbronn and Tolagen 1965; Clement 1979a). A group of oximes synthesized in the late sixties – the H-oximes – has, however, in the hands of several groups shown to be effective in the treatment of soman poisoning (Oldiges and Schoene 1970; Kepner and Wolthuis 1978; Wolthuis and Kepner 1978; Lipp and Dola 1980; Van Helden et al 1986; Shih et al 1991). The most effective of the H-oximes, HI-6, has also proven to be very effective in treatment of poisoning by several organophosphorus insecticides in man (Kušić et al 1991). The mechanism behind the effect of the oximes, and especially HI-6, is probably not exclusively enzyme reactivation, and remains to be elucidated (Heilbronn and Tolagen 1965; Clement 1981, 1983; Busker et al 1991; Van Helden et al 1991).

Pretreatment against organophosphate intoxication is relevant from a military point of view. The efficacy of reversible inhibitors of AChE, such as pyridostigmine and physostigmine, as prophylactic treatment against organophosphate intoxication havebeen evaluated in different animal models (Berry and Davies 1970; Heyl et al 1980; Lim et al 1989). The results from these experiments have shown that these carbamates are very effective as prophylactic agents, and may

be important as protection against the oxime-resistant nerve agent, soman. The reversible inhibitors of AChE temporarily renders a part of the AChE population insensitive to the organophosphate, with a subsequent reactivation of that part to physiologically active AChE. Pyridostigmine was used as prophylactic treatment by the Coalition Forces during Operation Desert Storm in the Gulf war in 1991. A retrospective study of the symptoms and disposition of 41650 soldiers who received pyridostigmine during this operation, has been performed through information by the personnel who provided medical support to the XVIII Airborne Corps (Keeler et al 1991). This study shows that severe adverse effects are relatively rare, and that this pyridostigmine regimen (oral administration of 30 mg pyridostigmine bromide every 8 hours) can be administered to virtually all soldiers under wartime conditions with little impairment of military performance.

2.2 Innervation of the airway smooth muscle

The nervous control of airway smooth muscle has in the past few years been shown to be much more complicated than previously anticipated. It involves several different levels of the nervous system and the action of many different neurotransmitters. This introduction will concentrate on peripheral mechanisms in the four different nervous systems that innervate the airway smooth muscle: (1) cholinergic excitatory, (2) nonadrenergic, noncholinergic (NANC) excitatory, (3) adrenergic inhibitory, and (4) NANC inhibitory.

The tension of the airway smooth muscle is mainly controlled by excitatory cholinergic input in most species including humans (Widdicombe 1963; Olsen et al 1965; Coburn 1987). The cholinergic neurons innervating the smooth muscle originates in the peripheral airway ganglia, and are dominantly innervated by vagal cholinergic neurons (Richardson 1979). Peripheral airway ganglia seems to coordinate neural input, not only to airway smooth muscle, but also to vascular smooth muscle, secretory cells, and epithelial cells in the airways (Coburn 1987). The muscarinic antagonist atropine inhibits contractions of airway smooth muscle induced by field stimulation, indicating that the postganglionic cholinergic neurons act on muscarinic cholinergic receptors located on the smooth muscle (Foster 1964). Tracheal tissues contain two different subtypes of muscarinic receptors (M_2 and M_3) as shown by both ligand binding studies (Madison et al 1987; Lucchesi et al 1990) and the existence of mRNA coding for both m_2 and m_3 receptors in tracheal extracts (Maeda et al 1988). Autoreceptors located

prejunctionally on postganglionic parasympathetic neurons in airways have been demonstrated in several species (Blaber et al 1985; Aas and Fonnum 1986; Faulkner et al 1986), and are most probably of the M_2 subtype (Blaber et al 1985; Minette and Barnes 1988; Aas and Maclagan 1990). In addition, there is evidence for M_1 receptors in the peripheral but not central airways in humans (Cazzola et al 1987b).

The presence of a NANC excitatory system in the airways was first demonstrated in the hilus bronchi of the guinea pig (Grundström et al 1981). Electrical field stimulation elicited atropine-resistant contractions which could be abolished by tetrodotoxin. These results have later been confirmed by other investigators (Lundberg and Saria 1982; Szolscányi and Barthó 1982; Lundberg et al 1983b). The NANC excitatory contraction is probably elicited by antidromic stimulation of sensory fibers of both vagal and spinal origin (Lundberg and Saria 1982; Lundberg et al 1983a,b; Dalsgaard and Lundberg 1984). Capsaicin, a principal pungent component found in hot peppers (Buck and Burks 1983), depletes substance P (SP) containing sensory terminals and has been used to study C-fiber afferents. Lundberg and Saria (1982) showed that the atropine-resistant component of the guinea pig airway contraction was resistant to hexamethonium but could be abolished by pretreatment with capsaicin both *in vivo* and *in vitro*. SP-like immunoreactivity is present in both central and peripheral terminals of nociceptive primary sensory neurons (Hökfelt et al 1975), and has been suggested to be the transmitter of these neurons (Leeman and Gamse 1981). Neurokinin A (NKA) is derived from the same gene as SP (Nawa et al 1983), and are consequently often co-localized. NKA is a more potent bronchoconstrictor *in vitro* than SP (Uchida 1987), as is calcitonin gene-related peptide (CGRP) (Palmer et al 1987). Accordingly, both NKA and CGRP may also be important regulators of bronchial tone. The ability of the sensory neurons to induce contraction when antidromically stimulated, indicate the presence of a local axon reflex arrangement in the peripheral part of this system (Kröll et al 1990).

Direct innervation of the airway smooth muscle by adrenergic nerves is sparse and is variable in different species (Weiner 1967; Mann 1971; Coburn and Tomita 1973). Receptor binding studies have shown a high density of β-receptors in lung of many species (Rugg et al 1978; Barnes et al 1980), including those species that seem to be devoid of adrenergic innervation. This indicates that β-receptors in airway smooth muscle are controlled mainly by circulating adrenaline. A high density of β-receptors in the epithelial cells, which upon stimulation release an epithelial relaxant factor (Goldie et al 1986), has also been shown (Barnes et al

1982). Contraction of airway smooth muscle in man mediated by α-receptors has been demonstrated both *in vivo* and *in vitro* (Simonsson et al 1972; Kneussl and Richardson 1978). The response were weak, however, and occurred only after blockade of β-receptors *in vitro*. In addition, innervation of the airway ganglia by adrenergic neurons has been shown (Jacobowitz et al 1973), indicating a possible adrenergic modulatory effect on excitatory input to the smooth muscle.

In addition to the relaxing response mediated by β-adrenergic receptors, there is also a nonadrenergic, noncholinergic (NANC) relaxing response (Foster 1964; Coburn and Tomita 1973; Richardson and Beland 1976). The NANC relaxatory response can also be induced by vagal stimulation, and is then sensitive to hexamethonium (Diamond and O'Donnell 1980; Yip et al 1981). The mediator of the nervous NANC relaxation response is most probably the neuropeptide vasoactive intestinal peptide (VIP) and the related gene product peptide histidine isoleucine (PHI) (for review see Barnes 1988). The neuropeptides are probably located to specific vesicles within cholinergic nerves in airway smooth muscle (Laitinen et al 1985a,b), and exert their action via stimulation of specific VIP receptors that is present on smooth muscle of large, but not small airways (Carstairs and Barnes 1986). VIP has been shown to act by two different mechanisms: direct pharmacomechanical relaxation of the airway smooth muscle (Ito and Takeda 1982) that is probably mediated via stimulation of adenylate cyclase (Lazarus et al 1986), and prejunctional inhibition of excitatory neurotransmitter release (Hakoda and Ito 1990).

2.3 Mediators affecting airway smooth muscle

There are several other mediators that have a pronounced effect on airways. It is beyond the scope of this introduction to discuss them all, but a few will be mentioned here. A great deal of research has been published concerning the effects of histamine, one ofthe main mediators released from mast cells. Histamine is known to interact with three receptor subtypes, which all are present in airways and seems to play different roles. Stimulation of the H_1 receptors produce bronchial smooth muscle contraction, while H_2 receptors seems to mediate the opposite effect, i.e. bronchial smooth muscle relaxation (Chand and DeRoth 1979), although the physiological role of this receptor is doubtful in some species including man (Tashkin et al 1982; Schachter et al 1982). A possible role of nervous reflex mechanisms in the airway constricting effect of histamine has been put forward, since block of parasympathetic nerve transmission inhibits the increase in

airway resistance caused by histamine aerosol (Malo et al 1982). This is probably due to an indirect stimulation of afferent fibers by histamine (Dixon et al 1979; Bergren and Sampson 1982). Recently, a possible role of the third histamine receptor subtype (H_3) in airways has been proposed. An inhibitory effect on cholinergic neurotransmission probably mediated by H_3 receptors has been demonstrated at the level of parasympathetic ganglia and postganglionic neurons in guinea pig trachea (Ichinose et al 1989) and at the level of postganglionic neurons in man (Ichinose and Barnes 1989a). Furthermore, stimulation of H_3 receptors in the airways of guinea pigs also inhibits vagally mediated NANC bronchoconstriction, probably by inhibition of peptide release (Ichinose and Barnes 1989b). Taken together, all this information on the effects of histamine on nerve mediated responses suggests that histamine may act as a modulator of excitatory neurotransmission in the airways.

Serotonin is released together with histamine from rat mast cells (Parratt and West 1957; Carlsson and Rizén 1969). Furthermore, serotonin has been shown to induce bronchoconstriction in rat (Aas and Helle 1982), as well as in guinea pig, calf and dog (Bhattacharya 1955; Offermeier and Ariëns 1966; Hahn et al 1978). Histamine does, however, not induce bronchoconstriction in rat (Aas and Helle 1982), suggesting that serotonin may be a more important pleural mast cell mediator influencing bronchial tone in this species. The effect of serotonin on airway tonus is partly inhibited by atropine (Bhattacharya 1955; Offermeier and Ariëns 1966; Islam et al 1974; Aas 1983), and potentiated by physostigmine (Aas 1983), indicating that the effect of serotonin is most probably mediated by both pre- and postjunctional receptors (Aas 1983; Macquin-Mavier et al 1991). Stimulation of irritant receptors may also, by a central vagal reflex, contribute to the bronchoconstriction induced by serotonin in dog (Hahn et al 1978) but probably not in guinea pig (Macquin-Mavier et al 1991). It has also been suggested that serotonin may play a role in the development of obstruction of the airways in patients with chronic obstructive pulmonary disease (Cazzola et al 1987a).

Physiological and anatomical evidence indicate that two amino acids, glutamate and γ-aminobutyric acid (GABA), are the quantitatively most important neurotransmitters in the brain (Curtis et al 1960; Curtis and Johnston 1974; Fonnum 1984, 1987). Recently evidence has accumulated indicating a possible role of these amino acids as neurotransmitters or neuromodulators in the peripheral nervous system (Jessen et al 1979; Giotti et al 1983; Jessen et al 1986; Moroni et al 1986; Yoneda and Ogita 1987). The excitatory neurotransmitter glutamate has been shown to enhance bronchial contractions induced by cholinergic neurons in rats

(Aas et al 1989). This response is specific for L-glutamate and seems to be mediated by a glutamate receptor different from the three subtypes that have been characterized in brain. A modulatory role of the inhibitory neurotransmitter GABA on bronchoconstriction has also been suggested. In the guinea pig GABA decreases the contraction of airway smooth muscle, probably by inhibition of release from both cholinergic and excitatory NANC nerve endings, via stimulation of $GABA_A$ and $GABA_B$ receptors, respectively (Tamaoki et al 1987; Belvisi et al 1989). The physiological significance of airway modulations by amino acids remains, however, to be elucidated.

3 GENERAL DISCUSSION

A better understanding of the different mechanisms involved in cholinergic neurotransmission is essential for improvement of the treatment of organophosphorus intoxication. The autonomic excitatory cholinergic neurotransmission in airway smooth muscle has in the present study been used as a model to elucidate both the mechanisms behind organophosphorus intoxication, and the mechanisms behind different regimes of treatment of this intoxication. Modulatory mechanisms, as shown for purines, have also been studied with the aim to explore possible unknown mechanisms in autonomic cholinergic neurotransmission in airways.

On the basis of the present investigation, this discussion will focus on the following points: the effect of soman on *in vitro* airway smooth muscle tonus (Chapter 3.1), the effect of pyridostigmine pretreatment and oxime treatment of soman poisoning (Chapter 3.2), calcium antagonists as potential therapeutics against the effect of soman on airways (Chapter 3.3), detoxification of soman by carboxylesterase (Chapter 3.4), and adenosine and cholinergic neurotransmission in airway smooth muscle (Chapter 3.5).

3.1 The effect of soman on in vitro airway smooth muscle tonus

The airway smooth muscle tonus is predominantly controlled by vagal cholinergic nerves in most species (Widdicombe 1963). Soman is believed to exert its acute toxic effect mainly by inhibition of AChE, and has, consequently, a substantial effect on the airway smooth muscle tonus in several species such as rat (Aas et al 1986, 1987), guinea pig (Paper I) and mongrel dog (Adler et al 1987). Airway contraction is probably the dominating mechanism behind the increased lung resistance induced by soman *in vivo* (Abbrecht et al 1989), although the finding that metacholine can induce vasodilation and increased respiratory tract secretions (Laitinen et al 1987) indicate that these factors may also be important. The mechanism underlying the effects of soman is probably inhibition of AChE activity with a subsequent increase in ACh concentration in the neuroeffector junction resulting in excessive stimulation of muscarinic receptors on the smooth muscle which induce contraction (Aas et al 1986; Adler et al 1987, 1992; Paper I). The fact that this effect, at higher concentrations of soman, was independent of other contraction inducing stimuli but still sensitive to atropine, indicate the presence of a constant leakage of ACh from these neurons. This is in accordance with

other studies, showing a high resting release of ACh from this tissue (Martin and Collier 1986).

Soman seems to affect the airway cholinergic system exclusively, since ED_{50} and maximal contraction induced by histamine in guinea pig were not changed by soman (Paper I). Other effects on the cholinergic system, apart from AChE inhibition can, however, not be excluded. It has recently been shown that soman, by a direct and reversible action on voltage-dependent ion channels, can affect the duration of calcium potentials in neurons in bullfrog sympathetic ganglia, and thereby their excitability (Heppner and Fiekers 1991). A possible direct effect of soman has also been shown in cat bladder sympathetic ganglia, where soman produced a membrane hyperpolarization that was insensitive to atropine, caffeine and phentolamine (Kumamoto and Shinnick-Gallagher 1990). Direct interactions of soman at the nicotinic ACh sites and at the adenosine receptor sites have also been shown (Albuquerque et al 1985; Lau et al 1988, 1991). Further investigations will be needed to elucidate which of these mechanisms that actively contributes to the toxic effects of soman and other organophosphates.

The apparent affinity of ACh was higher in guinea pig bronchial smooth muscle than in rat (Paper I; Aas et al 1986). Inter-species differences in sensitivity to ACh is not surprising since differences have been shown to exist even between different strains of guinea pigs (Mikami et al 1990). The guinea pig was also slightly different with respect to the sensitivity of AChE to soman. The guinea pig AChE activity was somewhat more sensitive to soman than AChE in rat bronchi and mongrel dog trachea (Paper I; Aas et al 1986; Adler et al 1987), further emphasizing the importance of exploring the effect of AChE inhibitors in several species before extrapolation to man.

The BuChE activities in rat and guinea pig bronchi, and mongrel dog trachea are also inhibited by soman (Aas et al 1986, 1987; Adler et al 1987; Paper I). This is of particular interest in airway tissue, since it has been shown that BuChE unlike in other tissues may play an important role in the breakdown of ACh in canine airway tissue (Adler and Filbert 1990; Adler et al 1991). Inactivation of ACh by BuChE seems primarily to be important when ACh is present in high concentrations, such as during high frequent stimulation or during exposure to anticholinesterases.

3.2 The effect of pyridostigmine pretreatment and oxime treatment of soman poisoning

Pretreatment with reversible inhibitors of AChE (carbamates) has been shown to protect against organophosphorus intoxication, probably by competition with the organophosphorus compounds of binding to the active site of the enzymes (Koster 1946; Koelle 1946). The interest in the efficacy of this pretreatment regimen was renewed by the fact that treatment with oximes was virtually without effect after intoxication with the organophosphate warfare agent soman (Heilbronn and Tolagen 1965; Clement 1979a). Emphasis has been placed on two carbamates, pyridostigmine and physostigmine, because they have been shown to be effective against the acute effects of soman in several mammalian species (Berry and Davies 1970; Heyl et al 1980; Lim et al 1989; Paper II).

Pyridostigmine is a quaternary compound, and does consequently not readily penetrate the blood-brain barrier, whereas the tertiary compound physostigmine readily crosses into the brain and thereby also protects against the central nervous system effects induced by organophosphates (Harris et al 1984). This means, on the other hand, that prophylactic use of physostigmine is associated with an increased risk of centrally mediated adverse reactions which can be avoided by the preferential use of pyridostigmine. Preclinical experiments on the sub-acute toxicity of pyridostigmine have revealed only mild gastrointestinal distress at therapeutic relevant doses administered orally, probably due to a slow uptake of this quaternary compound (Kluwe et al 1990). Furthermore, the duration of protection afforded by pyridostigmine after oral administration, which is the administration of choice for pretreatment of large groups, far exceeds that of physostigmine (Heyl et al 1980). Experiments evaluating the efficacy of modified carbamates have been published (Arnal et al 1990), and eventually, new drugs with more desirable properties than physostigmine and pyridostigmine will hopefully be available.

The protective effect of pyridostigmine pretreatment in different species has been shown to correlate well with the rate of decarbamylation in the same species (Ellin and Kaminskis 1989). It has, however, also been shown that continuous pyridostigmine pretreatment for several days is more effective against intoxication with soman in guinea pigs than a bolus of pyridostigmine administered 10 minutes before exposure to the nerve agent (Lim et al 1989). This difference indicates that other time demanding effects induced by carbamates may also be important in the protection afforded by carbamates. Continuous infusion of the

carbamate physostigmine has been shown to decrease the density of muscarinic receptors in guinea pigs (Lim et al 1988), thereby probably decreasing the sensitivity of target organs to elevated concentrations of ACh. This effect may also be important for the protection of the *ex vivo* cholinergic response in rat airways by pretreatment of pyridostigmine (Paper II), since inhalation of sub-acute concentrations of soman for 40 hours has been shown to decrease the density of muscarinic receptors in this tissue (Aas et al 1987). Some interesting recent findings indicates that pretreatment with the weak anticholinesterase, (+)physostigmine, protects against organophosphorus poisoning when used in concentrations that not produces detectable AChE inhibition (Kawabuchi et al 1988). One should therefore not exclude that direct effects of carbamates, not involving AChE, may actually also be therapeutically relevant (Albuquerque et al 1988).

In the classical treatment of poisoning by organophosphorus compounds, atropine is employed to counteract the stimulation of muscarinic receptors due to ACh accumulation, and an oxime is used to reactivate the inhibited enzyme (Lotti 1991). The effectiveness of different oximes is, however, dependent on both species and intoxicating agent. Oximes are effective only when the phosphylated AChE has not undergone "aging", i e dealkylation of the organophosphorus residue of the phosphylated enzyme. The rate of this reaction is primarily dependent on the type of organophosphate and, to a lesser degree, species (Ligtenstein 1984). Aging of soman is extremely rapid in many species, and most oximes have proven completely useless after intoxication with soman (Heilbronn and Tolagen 1965; Clement 1979a). The lack of efficacy is, however, not only due to rapid aging of the phosphylated enzyme. This was shown by the development of a group of oximes that were effective as reactivators of AChE inhibited by soman. This group of oximes – the H-oximes – synthesized by Hagedorn and coworkers (Freiburg, FRG) in the late sixties, have been shown to be effective in reversing the effects induced by many organophosphorus compounds, including soman, in several species (Sterri et al 1983; Alberts 1990; Clement 1992; Clement et al 1992; Paper II). HI-6 is one of the most promising oximes in this group of compounds, having a relatively low intrinsic toxicity (Kepner and Wolthuis 1978; Clement and Lockwood 1982; Kušić et al 1985; Liu and Shih 1990; Kušić et al 1991; Lundy et al 1992) and being the best reactivator of soman inhibited AChE (De Jong and Wolring 1980; Clement 1981; Harris et al 1990). It is also the most efficient against soman poisoning in several species (Kepner and Wolthuis 1978; Lipp and Dola 1980; Weger and Szinicz 1981; Clement and Lockwood 1982; Lundy and Shih 1983; Lundy et al 1992). In addition, therapeutic effect in humans has been shown after accidental and suicidal intoxication with different organophosphate

insecticides (Kušić et al 1991). The therapeutic effect of HI-6 against intoxication with soman is not limited to rodents, which have a slow rate of aging of the phosphylated enzyme (Wolthuis and Kepner 1978). HI-6 has also been shown to be effective in the treatment of soman intoxicated monkeys (Lipp and Dola 1980), indicating that some effects of HI-6 other than reactivation of AChE may also be involved.

Other actions of oximes, apart from enzyme reactivation, have been demonstrated in neuromuscular transmission models *in vitro* (Wolthuis et al 1981; Busker et al 1991). A part of the oxime-induced recovery of the neuromuscular transmission was in many preparations, particularly those from dog and rhesus monkey, not affected by a second (double) dose of soman, indicating that this part of the recovery was not due to reactivation of AChE. In addition, direct actions, only observed as long as the oxime was present in the organ bath, could also be observed. Addition of HI-6 30 minutes after exposure to soman did not induce significant recovery of the *in vitro* airway smooth muscle response in rat, indicating that other effects by HI-6 than enzyme reactivation may not be present in this tissue (Paper II). Several investigators have tried to explore the nature of these other effects observed, and several different effects of oximes have been reported, including ganglionic blockade (Lundy and Tremblay 1979), hemicholinium-3-like activity (Clement 1979b), and atropine-, and curare-like activity (Clement 1981). Which of these effects, if any, that contributes to the therapeutic effect remains to be elucidated.

It seems, however, that reactivation of enzyme activity is the main therapeutic mechanism when oximes are allowed to act before aging is completed (Van Helden et al 1991). A direct reaction with free organophosphate is also possible, especially if the oxime is administered before or immediately after exposure to the organophosphate. The product of this reaction, as well as the reaction between oxime and phosphylated enzyme, is a phosphylated oxime that may itself be a potent anticholinesterase (Hackley et al 1959; Rogne 1967), but it is usually relatively unstable and decompose rapidly (Fonnum 1975). The reaction of oxime with free organophosphate may give phosphylated oximes that are less liposoluble than the organophosphate, thereby also limiting the distribution of the anticholinesterase (Waser et al 1992).

The role of central effects of oximes in the therapy of organophosphate poisoning is uncertain, since most of these – like Toxogonin and HI-6 – are quaternary in structure, and cross the blood-brain barrier only to a small extent (Firemark et al

1964; Ligtenstein and Kossen 1983; Garrigue et al 1990). The therapeutic effects of HI-6 against poisoning with soman is primarily of peripheral origin, since intra-cerebro-ventricular administration of HI-6 failed to protect against subcutaneously injected soman in rats (Lundy and Shih 1983). Intraperitoneally injected HI-6 protects, on the other hand, against intra-cerebro-ventricular injected soman, which rapidly distributes to the periphery, as shown by a substantial inhibition of AChE in blood (Lundy and Shih 1983). These results do not exclude that oximes may have important central effects, but merely point out the importance of the peripheral effects of oximes for therapeutic efficacy.

Central effects of oximes in the therapy of organophosphate poisoning have been shown, in spite of their poor penetration of the blood-brain barrier. In rats, doses of approximately the LD_{10} of most organophosphates produce hypothermia of central origin that can be reduced by treatment with oximes (Meeter et al 1971; Wolthuis et al 1981). It has also been shown that respiratory failure following intoxication with low doses of soman is primarily due to inhibition of the respiratory centres in the brain (Wolthuis et al 1981), although these studies were performed on animals anaesthetized with barbiturates which may have rendered the respiratory centres particularly sensitive. Taken together, these results points toward that both central and peripheral effects of oximes may be important for their therapeutic effect, although the peripheral is probably the most important (Clement and Lockwood 1982).

3.3 Calcium antagonists as potential therapeutics against the effect of soman on airways

Calcium is an important intracellular messenger in all animal cells, and is essential for a variety of different biochemical and physiological functions. The intracellular concentration of calcium is regulated in a complicated fashion, involving voltage- and receptor-operated channels, calcium binding proteins, calcium ATP-ases, and ion exchange mechanisms (for review see Carafoli 1987). In the airways, calcium appears to have a central role in both release of the neurotransmitter ACh (Aas and Fonnum 1986; Paper III) and smooth muscle contraction (Coburn 1977; Creese and Denborough 1981; Paper III).

The intracellular calcium concentration is about 100–200 nM in the relaxed airway smooth muscle, and at this level of calcium the contractile apparatus is not activated. Under the influence of contractile agonists there is a sharp rise in

intracellular concentration of calcium to 0.5–1.0 μM (Murray and Kotlikoff 1991). There is only two sources from which this calcium can be derived: intracellular stores and/or the extracellular compartment. The high concentration of calcium in the extracellular compartment (1–2 mM), and thereby a large electrochemical gradient over the plasma-membrane, makes this an easily available source. An increase in the permeability for calcium would automatically induce influx of calcium, and increased intracellular concentration. Both voltage-operated channels (VOC) and receptor-operated channels (ROC) for calcium are present in plasmalemma of airway smooth muscle (Rodger 1987; Murray and Kotlikoff 1991).

The VOC's in airway smooth muscle, which are sensitive to organic calcium blockers such as verapamil and diltiazem, are responsible for the influx of extracellular calcium during stimulation with potassium or tetra-ethylammonium (Rodger 1987). The VOC's have been divided into three groups, L, T, and N, on the basis of their electrophysiological properties (Nowycky et al 1985; Fox et al 1987; Tsien et al 1988). The L channels are blocked by, and the T channels are relatively insensitive to, inorganic and organic antagonists (Nowycky et al 1985). The N channel, which is believed to be important for the triggering of neurotransmitter release, is insensitive to the organic calcium antagonists, but is blocked by the peptide ω-conotoxin GVIA (Kerr and Yoshikami 1984; Cruz et al 1987). There is, however, no evidence that supports the involvement of VOC's in contractions induced by physiological agonists such as cholinomimetics and histamine. The N channel calcium antagonist ω-conotoxin GVIA, on the other hand, effectively inhibits electrically elicited rat tracheal smooth muscle contractions but is ineffective as a blocker of carbachol induced contractions (Paper III), indicating that it inhibits the calcium influx into nerve endings but not airway smooth muscle.

ROC's differs from VOC's in that they are gated by agonist-receptor interactions, not wholly selective for calcium, voltage-dependent or -independent, and not readily inhibited by organic calcium antagonists (Bolton 1979). Agonist induced contraction of airway smooth muscle is not associated with action potential discharge, and contractions can be induced even in fully depolarised preparations (Suzuki et al 1976; Farley and Miles 1977). Influx of extracellular calcium in airway smooth muscle cells in response to agonists has not been demonstrated until very recently (Murray and Kotlikoff 1991). The results from that study indicates that calcium released from intracellular stores is responsible for the initiation of contraction, while the tonic phase is associated with influx of extracellular

calcium – probably via ROC's. The release of calcium from intracellular stores responsible for initiation of contraction in airway smooth muscle is most probably mediated by an intracellular messenger, 1,4,5-inositol trisphosphate (IP_3), that is rapidly formed in airway smooth muscle after stimulation with contractile agonist (Chilvers et al 1989; Langlands et al 1989).

The effect of organic calcium antagonists as therapeutics in asthma has been extensively investigated (for reviews see Löfdahl and Barnes 1986; Rodger 1990). Their effectiveness as asthmatic therapeutics is, however, limited by the fact that they only inhibit VOC's, which seems not to be involved in the excitation-contraction coupling (see above). There is also a considerable amount of evidence that shows that VOC- inhibiting calcium antagonists are ineffective as inhibitors of airway contractions induced by physiological agonists (for review see Rodger 1987). Development of antagonists of the ROC's may, however, prove to be important in the therapeutics of asthma, since they may be important for tonic contractions of the airways.

Despite the important role played by calcium in many biological processes, including neurotransmission, very little research has addressed the possible therapeutic effect of calcium antagonists in the treatment of organophosphorus poisoning. It has been shown that inclusion of verapamil or nifedipine enhance the protection afforded by oxime (2-PAM) and atropine against intoxication with diisopropylfluorophosphate (DFP) in mice, while diltiazem was without effect (Dretchen et al 1986). The mechanism behind the protection was suggested to be prevention of calcium-mediated depolarization of motor nerve terminals and subsequent excessive neurotransmitter release induced by organophosphates and prevention of bronchial smooth muscle contraction. The possible protective effect of the calcium antagonists on nerve terminals arise from the findings that anti-cholinesterases exert a multiplier effect by converting single action potentials into trains of repetitive action potentials with a subsequent increase in transmitter release (Standaert and Riker 1967; Riker and Okamoto 1969). The anti-epileptic drug phenytoin has been shown to antagonize this effect induced by physostigmine (Raines and Standaert 1966), probably by blockade of intracellular movement of calcium into nerve endings (Yaari et al 1979). The protective effect of calcium antagonists on the airway response is, however, probably small. This conclusion can be derived from the fact that organic calcium antagonists inhibits soman induced bronchial contractions only when applied in high concentrations (μM) (Paper III). The results from this study indicate, however, that airway contractions induced by soman may be more dependent on extracellular calcium

than agonist induced contractions. This may be due to a specific effect of soman, since physostigmine induced contractions were not affected by removal of calcium (Paper III). It is suggested that this difference may be due to soman-induced effects other than inhibition of AChE, possibly by alterations of the calcium channels in the plasmalemma. Effects of soman on VOC's have been shown in the bullfrog sympathetic ganglia preparation (Heppner and Fieker 1991). Furthermore, several organophosphates, including soman, are able to induce release of calcium from intracellular stores in, amongst others, PC12 cells (Kauffman et al 1990). This effect could not be induced by pyridostigmine or physostigmine. Nevertheless, the use of calcium antagonists to relief peripheral respiratory tract symptoms can probably not be achieved unless they are applied locally by inhalation.

3.4 Detoxification of soman by carboxylesterase during inhalation of soman

Organophosphates are detoxified by two different mechanisms: (1) hydrolysis in the liver (Little et al 1989), and (2) scavenging, i e irreversible binding of the toxic agent to a site where it does not induce a toxic reaction. The detoxifying role of the liver is illustrated by the difference in toxicity in rat depending on whether soman is injected subcutaneously or intraperitoneally (Sterri 1981). The detoxification in liver may therefore be important after oral poisoning by organophosphates. Exposure to nerve agents is, however, not primarily by the oral route, but rather through the respiratory system or through the skin. The rapid distribution of organophosphates to the organs make the detoxification by liver less important. The most efficient localization of a detoxification system is therefore in the blood, thereby limiting the distribution of active toxic agent to the organs. Detoxification of organophosphorus compounds by binding in equimolar proportions to the active site of the enzyme carboxylesterase (CarbE) has been known for several decades (Aldridge 1953; Jansz et al 1959). This enzyme, without any known physiological role, is present in most tissues in mammals, but is particularly abundant in rodent plasma (Myers 1952; Aldridge 1953; Augustinsson 1959). The role of plasma CarbE of rodents to explain their higher tolerance to low doses of soman compared to primates has been thoroughly investigated by Sterri and coworkers (Sterri and Fonnum 1989). They have, in a series of papers, shown that the tolerance to soman in rat increased with increasing plasma levels of CarbE (Sterri et al 1985a; Fonnum et al 1985), and that the difference in LD_{50} between different species correlates well with the difference in plasma concentration of CarbE (Sterri and Fonnum 1989). It has also been shown that the interspecies

differences in LD_{50} of soman can be abolished by pretreatment with a CarbE inhibitor (Maxwell et al 1987).

The plasma CarbE, especially the CarbE having 4-nitrophenyl butyrate hydrolytic activity, is more important for detoxification of subcutaneously injected soman than the liver and lung CarbE (Sterri et al 1985a). One of the primary routes of uptake of nerve agents is, however, through the respiratory tract, where more than 90% of an inhaled dose of the nerve agent sarin is absorbed cephalad of bronchi in rabbits and dogs (Ainsworth and Shephard 1961). The CarbE in airways is, accordingly, substantially inhibited after sub-acute inhalation of soman in rats, and may therefore function as an important scavenger of inhaled soman (Paper IV). Furthermore, lung CarbE is more inhibited after sub-acute inhalation of soman than after injection, and may therefore also constitute a more important barrier to soman than estimated on the basis of results from experiments with injections of nerve agents (Paper IV; Sterri et al 1985b). Nevertheless, the plasma CarbE in rat is probably the most important barrier to the distribution of soman to target organs also when inhalation is the route of uptake, since long-term subacute inhalation of soman inhibits erythrocyte AChE much more potently than AChE in brain and diaphragm (Paper IV).

The complete inhibition of BuChE in airways after sub-acute inhalation of soman indicate that this enzyme is also a barrier to the penetration of soman during inhalation (Paper IV). The relative importance of CarbE and BuChE depend on their concentration in the tissues, and the affinity to the toxic agent. However, the physiological role of BuChE in respiratory tissue (Adler and Filbert 1990; Adler et al 1991) means that BuChE cannot be considered as a detoxifying agent in this tissue, since the inhibition of BuChE in airways probably contribute to the respiratory symptoms during intoxication.

Injections of scavengers in animals have been shown to be effective as pretreatment against organophosphorus poisoning (Fonnum et al 1985; Raveh et al 1989; Ashani et al 1991). This may develop as a therapeutically important pretreatment in situations of warfare agent threat, provided that development and synthesis of easily handled, harmless and effective scavengers of nerve agents can be achieved.

3.5 Adenosine and cholinergic neurotransmission in airway smooth muscle

Intracellular adenosine is formed primarily by dephosphorylation of 5'-adenosine monophosphate and cleavage of adenylhomocysteine (for review see Henderson 1985). The intracellular level of adenosine is normally very low, but can increase dramatically during periods of high energy demand. Under this condition the intracellular concentration of adenosine may exceed the extracellular concentration, and adenosine can then diffuse via the nucleoside carrier down its concentration gradient and increase the extracellular concentration of adenosine resulting in stimulation of extracellular adenosine receptors.

Two different subtypes of adenosine receptors (A_1 and A_2) have been recognized (see however Ribeiro and Sebastião 1986), which both are antagonized by methylxanthines such as theophylline and caffeine. Stimulation of the different subtypes of receptors induce opposite effects on the enzyme adenylate cyclase, A_1 being inhibitory and A_2 stimulatory (Van Calker et al 1979). Some other effects mediated by A_1 receptors have also been reported, including modulation of the properties of calcium and potassium channels (Dolphin and Scott 1987; Trussell and Jackson 1987). The two receptors are differentiated on the basis of the order of potency of different adenosine analogues. The N^6-substituted adenosine agonists preferentially stimulates the A_1 receptor, while 5-substituted analogues are more potent than N^6-substituted on the A_2 receptor. The neuromodulatory effects of adenosine have been extensively investigated, and relates primarily to its inhibiting effect on neurotransmitter release (Ginsborg and Hirst 1972; Fredholm and Dunwiddie 1988), an effect that seems to be mediated by A_1 receptors.

Adenosine has been suggested to be one of the brains natural anticonvulsant (Dragunow and Faull 1988), since an adenosine antagonist has been shown to prolong epileptic seizures (Dragunow and Robertson 1987) and since there is a large rise in brain levels of adenosine during seizures (Winn et al 1980). Furthermore, the central stimulant actions of methylxanthines, such as caffeine and theophylline, may be due to their antagonistic properties on adenosine receptors (Snyder et al 1981). The fact that these effects are not induced by enprofylline, a methylxanthine with poor antagonistic potency on adenosine receptors (Persson et al 1981, 1982), is in favour of this assumption (Persson et al 1986).

Some direct evidence have also been published showing that adenosine analogues may, in addition to inhibition, actually mediate stimulation of neurotransmitter

release, both in the central nervous system (Spignoli et al 1984; Dolphin and Prestwich 1985; Brown et al 1990) and autonomic and somatic peripheral nervous system (Paper V; Correia-de-Sá et al 1991). The stimulating effect on release is probably mediated by an A_2 subtype receptor (Spignoli et al 1984; Brown et al 1990; Paper V; Correia-de-Sá et al 1991). Furthermore, it has been shown that augmented cAMP levels, which is the reported response to A_2 receptor activation (Van Calker et al 1979; Brown et al 1990), can enhance neurotransmitter release in brain slices (Dolphin and Archer 1983).

The effects of purine nucleosides and nucleotides on guinea pig trachea have been extensively investigated. The dominating effect is relaxation of the tracheal smooth muscle (Coleman 1976; Farmer and Farrar 1976; Brown and Collis 1982; Caparotta et al 1984; Collis et al 1989), although this relaxation is often preceded by a contraction at low concentrations of the agents (Coleman 1976; Farmer and Farrar 1976; Caparotta et al 1984; Ghai et al 1987; Farmer et al 1988). Dipyridamole, an adenosine uptake blocker, consistently enhanced the relaxation induced by both adenosine and ATP, indicating that the effect is mediated primarily by stimulation of extracellular adenosine receptors (Coleman 1976; Farmer and Farrar 1976; Darmani and Broadley 1986). The potency order of the adenosine agonists suggest that relaxation is mediated by stimulation of A_2 receptors, while contraction is most probably mediated by stimulation A_1 receptors (Ghai et al 1987; Farmer et al 1988).

Methylxanthines are inhibitors of the phosphodiesterase (PDE) enzymes, which are responsible for breakdown and inactivation of cyclic nucleotides within the cells. This inhibition was believed to be the mechanism behind the relaxatory effect of methylxanthines on airways. This view has, however, been challenged by the finding that methylxanthines relax airway smooth muscle at concentrations well below those needed for PDE inhibition (Lohmann et al 1977; Kolbeck et al 1979). It has therefore been suggested that the therapeutic effect of methylxanthines on airways may be due to displacement of adenosine from adenosine receptors, which occur at therapeutic concentrations of the methylxanthines. That would require, however, that adenosine receptor stimulation induce airway smooth muscle contraction, not relaxation which has shown to be the primary response (see above). Contraction responses induced by adenosine in resting airway tissue have been shown (Advenier et al 1982; Farmer et al 1988), as well as bronchoconstriction in asthmatic subjects (Cushley et al 1983). Furthermore, adenosine analogues enhance the cholinergic airway smooth muscle response in rabbit (Gustafsson et al 1986), dog (Sakai et al 1989) and rat (Paper V). The

mechanism of this cholinergic enhancement is probably stimulation of prejunctional adenosine receptors mediating enhanced release of ACh (Paper V). However, a methylxanthine with poor adenosine receptor antagonistic potency, enprofylline, has been shown to be a very potent airway relaxing agent (Persson et al 1981, 1982), suggesting that some other until now undiscovered effect may be responsible for the airway relaxing effect of methylxanthines.

Adenosine is generally considered as a smooth muscle relaxant in most tissues including airways (see above). Nevertheless, much attention has focused on adenosine as a possible mediator of bronchoconstriction in asthma. The interest in adenosine arouse from the finding that inhalation of adenosine, but not guanosine, induce airway constriction in asthmatic subjects exclusively (Cushley et al 1983). Furthermore, adenosine is a more potent constrictor of isolated human bronchi from asthmatics compared to non-asthmatics (Dahlén et al 1983). The potency order of adenosine and analogues on *in vitro* preparations suggest that the constriction is mediated by stimulation of an A_2 receptor (Holgate et al 1986). Supporting this mechanism *in vivo* is the finding that inhaled theophylline antagonizes the adenosine induced contraction (Cushley et al 1984), and that it is a 3-5 fold more potent antagonist of adenosine induced compared to histamine induced contractions (Cushley et al 1984). Dipyridamole, an adenosine uptake blocker, enhance the effect of inhaled adenosine (Cushley et al 1986), further supporting the view that adenosine exert its effect on extracellular receptors.

It has been shown, in some species, that adenosine analogues may enhance cholinergic neurotransmission in airways (Gustafsson et al 1986; Sakai et al 1989; Paper V). The mechanism behind this enhancement seems to be enhanced release of ACh mediated by stimulation of prejunctional adenosine receptors (Paper V). The *in vivo* adenosine induced bronchial constriction in asthmatics is, however, probably not mediated by the cholinergic system, since ipratropium bromide, a muscarinic antagonist, has very little antagonizing effect on this constriction (Mann et al 1985).

Several lines of evidence indicates that bronchoconstriction in human asthmatic subjects induced by inhaled adenosine most probably involves release of other mediators from mast cells in lung. IgE-dependent release of histamine from isolated human mast cells and basophils is enhanced by adenosine and adenosine analogues in an order of potency as indicated for A_2 receptors (Church et al 1983; Hughes et al 1984). The mast cell stabilizing agent, sodium cromoglycate, inhibits adenosine but not histamine induced bronchoconstriction (Cushley and

Holgate 1985). Adenosine has also, by a theophylline sensitive mechanism, been shown to enhance antigen induced bronchoconstriction and release of histamine in an isolated rat lung model (Post et al 1990).

Methylxanthines seems to have several different effects on airways, and unknown mechanisms, not involving adenosine antagonism or PDE inhibition, may also be important (Howell 1990). Their therapeutic use against the effects of organophosphates in airways are probably restricted to topical application, since most of the available xanthines would antagonize the neuroprotective effect of adenosine in the central nervous system. In light of the neuroprotective effect of adenosine, it would also be interesting to explore the possible use of a stable adenosine analogue against the effects of organophosphates on the central nervous system.

4 CONCLUSIONS

Soman, an organophosphate anticholinesterase, inhibits the serine esterases in respiratory tissues in rat and guinea pig. This inhibition affects the cholinergic control of the airway smooth muscle, and produce extensive and long-lasting bronchoconstriction. The mechanism behind the bronchoconstriction induced by soman is primarily cholinesterase (ChE) inhibition, although other mechanisms involving recruitment of extracellular calcium also may play a role.

Treatment with HI-6 produce a more effective and stable recovery of the rat airway *ex vivo* response after inhalation of soman, than treatment with Toxogonin. *In vitro* experiments show, however, the importance of immediate treatment after exposure. HI-6 is effective 10 min, but not 30 min, after exposure to soman, probably due to the rapid aging of soman inhibited ChE. Pretreatment with the carbamate pyridostigmine also produce a substantial recovery of the rat *ex vivo* cholinergic response. It is difficult to correlate these results with reactivation of ChE activity in homogenates from the respiratory tissues. The time dependence of the *in vitro* response of the oximes suggest, however, that enzyme reactivation is the dominant mechanism behind the recovery of the airway smooth muscle response. The discrepancy between physiological recovery and enzyme reactivation may be due to the fact that only a small part of the ChE that is responsible for the measured activity in the homogenates is actually physiologically important.

Organic calcium antagonists inhibits the *in vitro* contraction of rat bronchial smooth muscle induced by soman. The concentration range in which they are effective (μM) indicates, however, that a therapeutic benefit against the effect of soman on airways may be limited to topical application of the calcium antagonists. The peptide calcium antagonist, ω-conotoxin GVIA, inhibits cholinergic nerve induced, but not soman or carbachol induced, contraction of the airways, in support of the assumption that it antagonizes a calcium channel that is important for the triggering of neurotransmitter release.

The carboxylesterase (CarbE) in respiratory tissues may function as a scavenger of soman during inhalation. The plasma CarbE in rat is, however, probably a more important barrier to the distribution of soman to target organs, since long-term sub-acute inhalation of soman inhibits erythrocyte AChE much more potently than AChE in brain and diaphragm. Furthermore, this suggest that plasma may be the preferable situation of a exogenously introduced scavenger, also when inhalation is the route of uptake.

Adenosine may be an important modulator of cholinergic activity in rat airway smooth muscle. The potent adenosine analogue, NECA, enhance the cholinergic nerve mediated *in vitro* contractions, without affecting contractions induced by exogenously applied acetylcholine (ACh). The NECA induced cholinergic enhancement is inhibited by 8-phenyltheophylline (8-PT), a potent adenosine receptor antagonist. The evoked release of tritium in rat airway preparations pre-loaded with ^{3}H-choline is also enhanced by NECA in an 8-PT sensitive manner, suggesting that the effect of NECA is mediated via prejunctional adenosine receptors which enhance evoked cholinergic release upon stimulation.

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Paper I

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THE EFFECT OF ACETYLCHOLINESTERASE-INHIBITION ON THE TONUS OF GUINEA-PIG BRONCHIAL SMOOTH MUSCLE

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Abstract—The irreversible acetylcholinesterase inhibitor soman (O-[1,2,2-trimethylpropyl]-methylphosphonofluoridate) induced contraction of guinea-pig primary bronchial smooth muscle. The apparent affinity (ED_{50}) of acetylcholine (ACh) was altered from control value of 12 μM to 0.3 μM following exposure of the bronchial smooth muscle to 14 μM soman for 15 min *in vitro*. The ED_{50} of the cholinergic agonist carbachol was not changed even when the acetylcholinesterase (AChE) activity was inhibited completely. The intrinsic activity (α) of ACh and carbachol was not significantly changed after exposure to soman for 15 min. The results demonstrate that the effect of soman is only due to its anticholinesterase activity. Furthermore, the contraction induced by histamine was not altered by concentrations of soman which increase the cholinergic stimulation. This indicates that histamine does not induce contraction of bronchial smooth muscle in guinea pig through the release of ACh or by modulation of muscarinic receptors. Furthermore, soman also inhibited the carboxylesterase activity in the primary bronchi. In respiratory tissue this group of enzymes may have a major protective function, due to their ability to bind several organophosphorus compounds. Compared to studies performed on other species, this study shows that guinea-pig bronchi are very sensitive to the AChE-inhibitor soman. Therefore, exposure to very low concentrations of AChE-inhibitors may induce contraction of bronchial smooth muscle.

The acute toxic effects of organophosphates are primarily mediated by an irreversible inhibition of acetylcholinesterase (AChE), resulting in local accumulation of acetylcholine (ACh). The extensive use of organophosphates increases the risk of human exposure. The important route of exposure is by inhalation and following exposure the cause of death is anoxia due to a combination of factors; severe bronchoconstriction, excess accumulation of bronchial and salivary gland secretions, weakness or paralysis of the accessory muscles of respiration and sudden respiratory paralysis.

Previous studies have shown that the organophosphorus compound soman (O-[1,2,2-trimethylpropyl]-methylphosphonofluoridate) has a substantial effect on the rat bronchial smooth muscle [1, 2]. Inhibition of AChE-activity by soman exposure *in vitro* or by inhalation exposure *in vivo* increases the synaptic concentration of ACh and thereby the stimulation of muscarinic receptors with subsequent contraction of bronchial smooth muscle. Similar results have been found in tracheal smooth muscle isolated from adult mongrel dogs, where soman potentiated the cholinergic stimulation by inhibition of AChE-activity. This potentiation was not due to stimulation of muscarinic receptors [3]. There is an obvious variability in the innervation of the airways and lung among species of animals, and extrapolation from one species to another, with regard to either physiologic responses or anatomic distribution of the nerves should be made with caution [4]. The small and large airways from guinea-pig, dog and man respond with contraction on exposure to histamine [5]. Airway smooth muscle from the rat, on the other hand, is not sensitive to histamine, but is contracted by 5-hydroxythryptamine (5-HT) [6]. This is in agreement with the results showing that the anaphylactoid reaction in rat is primarily mediated by 5-HT and not by histamine [7]. Previously, in rat and dog the classical mast-cell degranulating agent 48/40 has shown to produce a severe anaphylactoid reaction, but only a negligible effect is seen in guinea pig and man [8].

To elaborate on the effect of soman on the airway smooth muscle we have used the primary bronchi from the guinea pig. The guinea-pig airways, unlike rat and dog airway smooth muscle, have been shown to have many similarities with the human airways where histamine in addition to ACh play an important role as a mediator of contraction [5].

Previously soman has been shown to inhibit AChE-, ChE- and carboxylesterase (CarbE) activities in respiratory tissue as well as in other peripheral tissues in rat following exposure to soman by inhalation [2, 9]. In respiratory tissue CarbE may have a major protective function, due to their ability to bind several organophosphorus compounds [10, 11]. This may be of great importance since the lung is the primary route of entry for these compounds.

The aim of the present work was to study the effect of soman on the cholinergic nervous system in guinea-pig bronchial smooth muscle. Secondly, it was of interest to investigate whether soman altered the contraction induced by histamine. A third objec-

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tive was to determine the importance of the CarbE enzymes in the protection of AChE in bronchial smooth muscle.

MATERIALS AND METHODS

Animals

Male guinea pigs (MOL:DH) within the weight range 200–300 g were used. The animals were given a standard laboratory diet and given water *ad lib*. The guinea pigs were without symptoms of infections in the respiratory system. They were examined at the National Institute of Public Health, Oslo, Norway.

Chemicals

Acetylcholine chloride, atropine sulphate and histamine dihydrochloride were purchased from Norsk Medisinal Depot, Oslo, Norway. Carbachol and tetrodotoxin was from Sigma Chemical Co. (Poole, U.K.).

Soman (O-[1,2,2-trimethylpropyl]-methyl-phosphonofluoridate, assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory.

Physiological methods

Following decapitation and dissection the left and right primary bronchi were mounted in parallel and contraction was measured as a reduction of the diameter of the airway [6]. The thermostatically controlled organ-bath contained Krebs buffer (50 ml, 37°) of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; glucose, 11.1 (pH = 7.4). The solution was gassed with 95% O_2 + 5% CO_2. For electrical stimulation the bronchi were mounted between platinum electrodes and stimulated by a Grass S88 Stimulator. The preparations were given a preload of 1.6 g and equilibrated for 60 min before the start of the experiments. The preload of 1.6 g was selected because the contraction response to a maximal stimulation with carbachol was rapid and gave a high and constant level of contraction. Carbachol was used as the reference agonist ($\alpha = 1.0$) since carbachol was most potent. The contractions were recorded isometrically by Grass Force Displacement Transducers (FT 03C) and monitored on a Grass Polygraph (Model 7) fitted with amplifiers (7 P 1A).

Acetylcholine, carbachol and histamine were added by cumulative application, since there were no differences in the apparent affinities (ED_{50}) when the agonists were added by single or by cumulative application.

Soman was added to the *in vitro* preparation and left for 15 min before it was removed by washing with the Krebs buffer.

Biochemical methods

Determination of acetylcholinesterase (AChE) and pseudocholinesterase (ChE) activities. Following the physiological experiments (approximately 6 hr) the guinea-pig bronchi were frozen (−20°) and later homogenized (10% w/v) in 20 mM sodium–potassium phosphate buffer (pH = 7.4) (glass/glass homogenizer, 20 strokes, 720 rpm, ice cold) before the enzyme activity determinations. Total ChE-activities were determined by the radiochemical method of Sterri and Fonnum [12] at 30°. AChE-activity was measured after inhibition of ChE-activity with ethopropazine [13].

Determination of carboxylesterase (CarbE) activity. The activity of CarbE, which hydrolyze 4-nitrophenylbutyrate, was determined at 30° by a spectrophotometric method [14]. The assay mixture consisted of 0.1 M sodium phosphate buffer pH = 7.8, 2 mM 4-nitrophenylbutyrate and tissue homogenate in a total volume of 3.0 ml. The stock solution of 4-nitrophenylbutyrate was 0.6 M in acetone. Optical absorbance of 4-nitrophenol at 400 nm was followed in a Beckman DU-50 Spectrophotometer fitted with kinetics Soft-Pack module, with the assay mixture omitting the tissue homogenate as reference. A molar absorption coefficient of 17,000 M^{-1} cm^{-1} was used [15]. Protein content was determined by the Method of Lowry [16].

Statistics

Data are given as mean ± SEM. Statistical analyses were performed with the Student's *t*-test (two-tailed).

Table 1. The effect of soman on acetylcholinesterase (AChE), pseudocholinesterase (ChE) and carboxylesterase (CarbE) activities in the guinea-pig primary bronchial smooth muscle

Soman (M)	Enzyme activities (μmol × min^{-1} × mg $protein^{-1}$ ± SEM) AChE	ChE	CarbE	N
0	$3.5 \pm 0.6 \times 10^{-3}$ (100 ± 17)	$2.8 \pm 0.5 \times 10^{-3}$ (100 ± 18)	$4.1 \pm 0.3 \times 10^{-2}$ (100 ± 7)	5
1.4×10^{-9}	$4.4 \pm 0.7 \times 10^{-3}$ (126 ± 20) ns	$2.2 \pm 0.2 \times 10^{-3}$ (76 ± 8) ns	$2.7 \pm 0.4 \times 10^{-2}$ (66 ± 10)*	8
1.4×10^{-8}	$5.4 \pm 1.3 \times 10^{-4}$ (16 ± 4)**	$3.0 \pm 0.4 \times 10^{-4}$ (11 ± 1)**	$2.4 \pm 0.3 \times 10^{-2}$ (57 ± 7)**	4
1.4×10^{-7}	$1.7 \pm 0.4 \times 10^{-4}$ (5 ± 1)**	$1.9 \pm 0.3 \times 10^{-4}$ (7 ± 1)**	$1.3 \pm 0.3 \times 10^{-2}$ (32 ± 7)**	6
1.4×10^{-6}	0	0	$3.6 \pm 0.1 \times 10^{-3}$ (9 ± 1)**	4
1.4×10^{-5}	0	0	0	5

Acetylcholinesterase (AChE), pseudocholinesterase (ChE) and carboxylesterase (CarbE) activities in guinea-pig primary bronchi after treatment with soman *in vitro*. The bronchi were exposed to soman for 15 min in a physiological buffer prior to recording of the contraction induced by cholinergic and histaminergic stimulation. Following the physiological experiments the enzyme activities were measured as described in Materials and Methods. The results are expressed as mean ± SEM and resulted in differences from controls at **$P < 0.01$, *$P < 0.05$, ns $P > 0.05$. The results in parenthesis are in per cent of control.

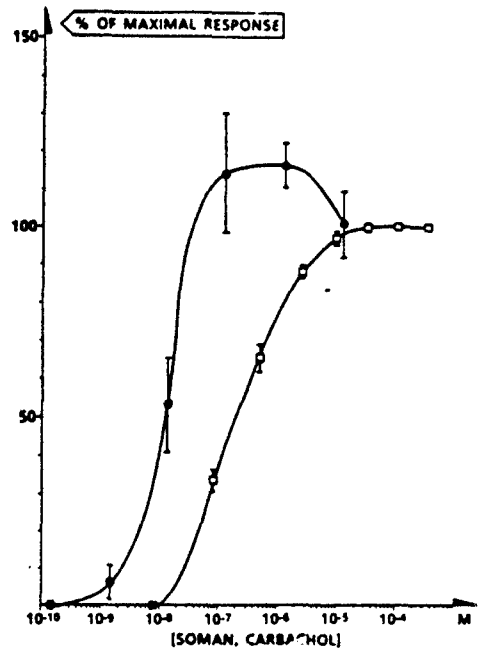

Fig. 1. Concentration–response curves for soman (●, N = 6) and carbachol (□, N = 37) on the isolated primary bronchi from guinea pig. The responses to soman and carbachol are mean ± SEM of N experiments and plotted in per cent of the maximal response to carbachol before exposure to soman.

RESULTS

The results show that guinea-pig primary bronchi were very sensitive to soman since concentrations of soman in the nanomolar range enhanced the contractions induced by ACh and electrical stimulation. Soman present alone in concentrations lower than 1 nM produced no contractions and maximal response was reached at approximately 100 nM of soman (Fig. 1). The response to application of soman was rapid and the contraction was irreversible in the absence of a muscarinic antagonist. The maximal contraction induced by soman was in the same range as was seen for carbachol (Fig. 1). The spontaneous increase in muscle tension induced by soman was antagonized by atropine and the concentration-dependent response to soman correlated well with the inhibition of AChE-activity (Table 1). Complete inhibition of AChE-activity occurred after *in vitro* exposure to a soman-concentration of 140 nM for 15 min.

The intrinsic activities of ACh and carbachol were in the same range, whereas the intrinsic activity of histamine was larger ($\alpha = 1.25 \pm 0.1$, N = 9) compared to carbachol ($\alpha = 1.0$). There was no alteration of the apparent affinity (ED_{50}) to carbachol (Table 2) or histamine (Table 2) following exposure to soman (1.4 nM–14 μM). In comparison, soman substantially reduced the ED_{50} of ACh from 12 μM to 0.3 μM (Table 2). A low concentration of soman (14 nM) enhanced the response to cholinergic stimulation of the guinea-pig bronchial smooth muscle shown by

Table 2. The effect of soman on apparent affinity (ED_{50}) and intrinsic activity (α) of acetylcholine, carbachol and histamine in guinea-pig bronchi

Soman (M)	ED_{50} (M)			α			α'		
	Acetylcholine	Carbachol	Histamine	Acetylcholine	Carbachol	Histamine	Acetylcholine	Carbachol	Histamine
0	$1.2 \pm 0.6 \times 10^{-7}$ (32)	$2.6 \pm 0.4 \times 10^{-7}$ (31)	$2.1 \pm 0.3 \times 10^{-4}$ (30)	1.00(32)	1.00(31)	1.00(30)	1.00(32)	1.00(31)	1.00(30)
1.4×10^{-9}	$2.8 \pm 2.3 \times 10^{-7}$ (4)	$1.3 \pm 0.8 \times 10^{-7}$ (6)	$3.8 \pm 0.6 \times 10^{-4}$ (5)	$1.21 \pm 0.02^{**}$ (4)	$1.14 \pm 0.10^{**}$ (6)	$1.14 \pm 0.12^{**}$ (5)	$1.23 \pm 0.08^{**}$ (4)	$1.21 \pm 0.09^{**}$ (6)	$1.20 \pm 0.13^{**}$ (5)
1.4×10^{-8}	$5.5 \pm 1.0 \times 10^{-7**}$ (5)	$2.1 \pm 1.3 \times 10^{-7}$ (6)	$1.8 \pm 0.4 \times 10^{-4}$ (7)	$1.41 \pm 0.16^{**}$ (5)	$0.98 \pm 0.17^{**}$ (6)	$0.86 \pm 0.14^{**}$ (7)	$1.87 \pm 0.27^{**}$ (5)	$1.51 \pm 0.23^{**}$ (6)	$1.79 \pm 0.12^{**}$ (7)
1.4×10^{-7}	$2.0 \pm 0.5 \times 10^{-7**}$ (7)	$1.5 \pm 0.5 \times 10^{-6**}$ (7)	$4.2 \pm 0.4 \times 10^{-4}$ (5)	$0.71 \pm 0.05^{**}$ (7)	$0.31 \pm 0.03^{**}$ (7)	$0.29 \pm 0.09^{**}$ (5)	$1.73 \pm 0.13^{**}$ (7)	$1.43 \pm 0.18^{**}$ (7)	$1.02 \pm 0.13^{**}$ (5)
1.4×10^{-6}	$2.9 \pm 1.2 \times 10^{-7**}$ (8)	$1.6 \pm 0.4 \times 10^{-6**}$ (6)	$2.7 \pm 0.8 \times 10^{-4}$ (6)	$0.70 \pm 0.08^{**}$ (8)	$0.58 \pm 0.11^{**}$ (6)	$0.30 \pm 0.07^{**}$ (6)	$1.76 \pm 0.13^{**}$ (8)	$1.35 \pm 0.15^{**}$ (6)	$1.07 \pm 0.11^{**}$ (6)
1.4×10^{-5}	$3.0 \pm 1.4 \times 10^{-7**}$ (6)	$1.1 \pm 0.3 \times 10^{-6}$ (6)	$3.3 \pm 2.0 \times 10^{-4}$ (5)	$0.34 \pm 0.05^{**}$ (6)	$0.16 \pm 0.03^{**}$ (6)	$0.21 \pm 0.08^{**}$ (5)	$1.46 \pm 0.14^{**}$ (6)	$1.16 \pm 0.10^{**}$ (6)	$1.14 \pm 0.08^{**}$ (5)

The intrinsic activities of acetylcholine, carbachol and histamine were recorded before and after exposure to soman. α' is the intrinsic activity of the respective agonist after exposure to soman, while α' is the intrinsic activity of the agonist including the activity of soman. The α'- and α'-values are relative to control in the absence of soman, which is given an $\alpha = 1.00$. The results are expressed as mean ± SEM of the number of experiments shown in brackets and resulted in differences from control at $^{**}P < 0.01$, $^{*}P < 0.05$, $^{ns}P > 0.05$.

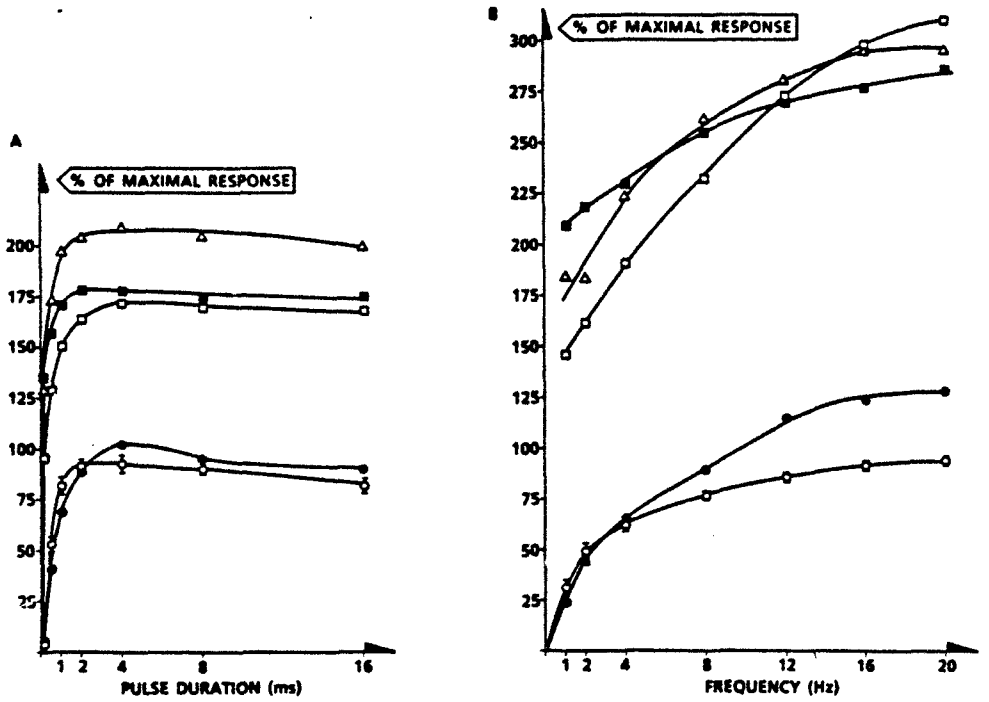

Fig. 2. Effect of electrical stimulation of the isolated guinea-pig primary bronchial smooth muscle. Part A shows the effect of varying the pulse duration (0.1–16 msec) and keeping the pulse frequency constant (10 Hz). Part B shows the effect of varying the pulse frequency (1–20 Hz) with a constant pulse duration (6 msec). The responses are mean ± SEM of N experiments and plotted in per cent of control stimulation without soman (○, N = 29); soman; 1.4 nM (●, N = 5), 14 nM (□, N = 3), 140 nM (■, N = 7), 1.4 μM (△, N = 6).

the decrease in ED_{50} of ACh from 28 to 0.55 μM.

Soman had no effect on the contraction induced by maximal stimulation with ACh, carbachol or histamine when the spontaneous increase in muscle tension induced by soman was included in the response (α'') (Table 2). However, exposure to soman reduced the maximal contraction (α') induced by the agonists when the intrinsic activity was not corrected for the soman-induced contraction (Table 2).

As shown in Table 1, in addition to inhibit AChE-activity soman also decreased the activities of pseudocholinesterases (ChE) and carboxylesterases (CarbE) in the guinea-pig bronchi (Table 1). There was a concentration-dependent inhibition of the ChE- and CarbE-activities, with an inhibition of 89 and 43% respectively following exposure to 14 nM of soman for 15 min. The most pronounced decrease in enzyme activities occurred following exposure to 14 nM of soman. A complete inhibition of AChE- and ChE-activities was seen following 15 min exposure to approximately 140 nM.

Low concentrations of soman (14 nM) also enhanced the efficacy of electrical field stimulation of the muscle (Fig. 2). It was previously shown that electrical stimulation release ACh and thereby stimulate to contraction [6]. It was blocked by atropine (1 μM) and tetrodotoxin (1 μM) (not shown). High concentrations of soman (micromolar range), however, induced contraction and therefore reduced the contraction response following electrical field stimulation. Together soman and electrical field stimulation enhanced the tension of guinea-pig bronchi severalfold (Fig. 2). Exposure to soman enhanced the contraction induced by increasing both the frequency and the duration of stimulation.

DISCUSSION

Soman is a specific irreversible AChE-inhibitor [17] and therefore a good model substance for studying the effect of AChE-inhibitors in the respiratory system. The present results show a concentration-dependent inhibition of AChE- and ChE-activities and a good correlation between the esterase-activities and the tonus of the guinea-pig bronchial smooth muscle. The results are in good agreement with previous results showing substantial effects of soman on the cholinergic nervous system in rat bronchial smooth muscle [1] and tracheal smooth muscle from mongrel dogs [3]. The inhibition of the AChE-activity was, however, more pronounced in the guinea-pig bronchial smooth muscle, indicating a higher sensitivity of AChE to soman in this species. A higher apparent affinity (ED_{50}) of ACh in guinea-pig bronchial smooth muscle compared to rat [1] was also seen, giving further evidence for the higher sensitivity of the cholinergic muscarinic receptors in

guinea-pig bronchial smooth muscle to cholinergic stimulation. It is therefore of importance to explore the effect of AChE-inhibitors in several species, before extrapolation of the sensitivity in man is drawn.

The results of this study have clearly shown that it is only the cholinergic nervous system which is altered by soman. Since the effect of soman was completely blocked by atropine, there was no evidence of stimulation of muscle cells *per se*. Furthermore, since the fact that soman did not change the ED_{50} or the maximal contraction induced by histamine, the result implies that neither soman nor ACh interact with histamine receptors. These results clearly show that although histamine is an important mediator of contraction in guinea-pig and human airways [5], histamine does not modulate the effect of ACh, at least not in the guinea-pig bronchial smooth muscle.

In the presence of soman (140 nM–14 μM) a decrease in the intrinsic activity (α') of ACh, carbachol and histamine was seen (Table 2). The results with ACh and carbachol are in agreement with results on the bronchi isolated from albino rats [1]. The reduced effects of ACh, carbachol and histamine in the presence of soman is probably due to inhibition of AChE-activity and the subsequent accumulation of spontaneously released ACh followed by stimulation of muscarinic receptors. The release of endogenously synthesised ACh is shown during electrical stimulation. The intrinsic activity (α'), which is the summation of the contraction induced by both the agonists and soman, was not different from control. This implies that soman does not enhance the maximal contraction of bronchial smooth muscle induced by ACh, carbachol and histamine, but only increases the sensitivity to cholinergic stimulation.

The data from electrical stimulation support the hypothesis that the soman-induced contraction was mediated by accumulation of ACh. Previously electrical stimulation has been shown to induce release of ACh from rat bronchial smooth muscle [6] and it has also been shown to be inhibited by tetrodotoxin and atropine. The most pronounced effect on electrical stimulation was seen following exposure to 14 nM soman. This correlates well with the inhibition of AChE-activity which was substantial at this concentration. A similar enhancement of electrical stimulation was seen in rat [1], although the effect during high frequency stimulation was much smaller. These results may also indicate that guinea-pig bronchi is more sensitive to AChE-inhibition than rat bronchi. As in the rat bronchial smooth muscle, at high concentrations (micromolar range) of soman, a sustained contraction of the guinea-pig bronchial smooth muscle was seen. This is due to the complete inhibition of AChE-activity and accumulation of spontaneously released ACh and a following stimulation of muscarinic receptors.

Previously carboxylesterases (CarbE), which is a group of several isoenzymes, has been shown to bind organophosphorus compounds irreversibly. It is therefore suggested that these enzymes can act as scavengers and reduce the ability of organophosphates to inhibit AChE [10, 11, 18, 19]. Following exposure of rats to soman *in vivo* [9, 20] or rat bronchial smooth muscle *in vitro* [1] both AChE- and CarbE-activities were inhibited to a large extent. CarbE were, however, less inhibited, which means that soman reached the target enzyme, AChE, although CarbE was present. This may indicate that CarbE play a less important role in respiratory tissue compared to other tissues in preventing soman from inhibiting the AChE-activity.

In conclusion, the results of this study show that guinea-pig bronchial smooth muscle AChE-activity is more sensitive to inhibition by soman than AChE in rat bronchi and mongrel dog tracheal smooth muscle. Furthermore, the results show that inhibition of AChE-activity by soman does not enhance the guinea-pig bronchial smooth muscle sensitivity to histamine. The CarbE enzymes does not prevent soman from inhibiting the AChE-activity in guinea-pig bronchi and they may therefore constitute a poor protection to soman in the respiratory organ.

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Paper II

THE EFFECT OF PYRIDOSTIGMINE PRETREATMENT, HI-6 AND TOXOGONIN® TREATMENT ON THE RAT TRACHEAL SMOOTH MUSCLE RESPONSE TO CHOLINERGIC STIMULATION AFTER ORGANOPHOSPHORUS INHALATION EXPOSURE

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ABSTRACT

The *ex vivo* contraction response of the rat tracheal smooth muscle was examined after 10 min *in vivo* inhalation of soman and/or pretreatment with pyridostigmine and/or post-exposure treatment with HI-6 ([[[(4-aminocarbonyl)pyridinio]methoxy]methyl]-2[(hydroxyimino) methyl]pyridinium dichloride) or Toxogonin® (1,1'-[oxybis-(methylene)]bis[4[(hydroxyimino) methyl]-pyridinium] dichloride). *In vivo* pretreatment with pyridostigmine was achieved by subcutaneous (sc) implantation of an osmotic pump that delivered pyridostigmine continuously (0.01 mg/hr) in the neck region of the rat 18 hrs before soman exposure. The *ex vivo* cholinergic tracheal smooth muscle response increased the first 60 min after soman exposure in animals pretreated with pyridostigmine. The response in pyridostigmine pretreated animals was about 60% of control, compared to 15% of control without pyridostigmine pretreatment. Pyridostigmine pretreatment also produced significant recovery of the cholinesterase (ChE) activity in plasma, but not in trachea and lung. Intraperitoneal (ip) injection of HI-6 or Toxogonin® (50 mg/kg), immediately after 10 min inhalation exposure to soman, also significantly improved the *ex vivo* cholinergic contraction response of the trachea (decapitation 15 min after oxime administration). The recovery of the physiological response with Toxogonin® was, however, not stable. HI-6 was superior to Toxogonin® with respect to the initial airway contraction response, and the response increased up to a stable level not significantly different from control. There was no significant reactivation of the ChE activity after treatment with the oximes. Combination of pyridostigmine prophylaxis and oxime treatment enhanced the recovery of the tracheal contraction response and the ChE activity in the trachea compared to treatment with oximes alone. Experiments with *in vitro* exposure to soman followed by washout and addition of oximes were also performed. The results show that both oximes effectively reestablish the tracheal response when administered 10 min, but not 30 min, after soman. The effect of Toxogonin® was, however, contrary to the effect of HI-6, not stable. These results correspond to the *in vivo* exposure experiments.

The results from this study indicate that HI-6 produces a more potent recovery of an *ex vivo* peripheral cholinergic response than Toxogonin® after 10 min inhalation exposure to soman.

Organophosphorus inhalation - Tracheal smooth muscle contraction - Oxime treatment - Pyridostigmine prophylaxis

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INTRODUCTION

The acute toxic effect of organophosphorus compounds are primarily due to an irreversible inhibition of acetylcholinesterase (AChE) and a subsequent accumulation of acetylcholine (ACh) in the synaptic cleft. The primary route of uptake of the organophosphorus nerve gases is by inhalation, and the symptoms include bronchoconstriction, increased bronchial secretion, and weakness and paralysis of respiratory muscles. Earlier studies have shown that inhalation of the organophosphonate soman potently inhibits the AChE of the rat respiratory system (Aas *et al.* 1985, 1987; Walday *et al.* 1991).

The conventional treatment of organophosphorus intoxication includes the muscarinic antagonist atropine plus an oxime. Oximes are capable of nucleophilic attack on phosphylated AChE, giving free enzyme and phosphylated oxime (Wilson and Ginsburg 1955). Poisoning with most OPs is treatable with this combination, but intoxication with soman is resistant to treatment with most oximes. HI-6 (fig. 1), a member of a new class of oximes synthesized in the late 60's by Hagedorn, has been shown to have therapeutic benefit against poisoning with soman in several species (Lipp and Dola 1980; Boskovic *et al.* 1984; Van Helden *et al.* 1986; Shih *et al.* 1991). HI-6 seems to act primarily in the periphery by reactivation of phosphylated cholinesterase before dealkylation (aging) has occurred (Sterri *et al.* 1983; Shih *et al.* 1991), although some recently published results have directed the attention to other possible direct effects of this oxime (Hamilton and Lundy 1989; Van Helden *et al.* 1991).

The uncertainty regarding the effectiveness of oximes against intoxication with soman called for the exploration of alternative treatments. It has been known for several decades that pretreatment with carbamates can protect the AChE activity against irreversible inhibition with OPs (Koster 1946; Koelle 1946). Carbamate pretreatment also produced a major breakthrough in the therapy of poisoning with soman (Berry and Davies, 1970). Pyridostigmine is a quaternary amine carbamate that is currently used to treat myasthenia gravis and has also been shown to be protective against soman poisoning when administered as a prophylactic agent (Gordon *et al.* 1978; Dirnhuber *et al.* 1979; Leadbeater *et al.* 1985; Lennox *et al.* 1985).

The object of the present study was to investigate the effect of *in vivo* pyridostigmine prophylaxis and/or *in vivo* oxime (HI-6 or Toxogonin®, fig. 1) treatment on an *ex vivo* peripheral cholinergic response in the rat after inhalation of soman. Reactivation of incubated enzyme was also examined for correlation to physiological response.

METHODS

Animals

Male Wistar rats (200-300g) (from Møllegaard, Copenhagen, Denmark) were kept in standard laboratory cages for 1-2 weeks with free access to standard laboratory diet and water before start of the experiment. The light/dark cycle was 12 hrs, relative humidity 45-55% and temperature 22-25°C. The animals were kept on sawdust which was replaced daily.

Chemicals

Carbachol, pyridostigmine bromide, bovine serum albumin (fraction V) and indomethacin were purchased from Sigma Chemical Co., Poole, England. [1-^{14}C]Acetylcholine chloride ([^{14}C]ACh) was from Amersham, Buckinghamshire, England; acetylcholine iodide (ACh) and 1.2-propanediol from Fluka AG, Buchs, Switzerland; Hypnorm® from Janssen, Beerse, Belgium; Dormicum® from Roche, Basel, Switzerland; tri-ortho-cresyl phosphate (TOCP) from K&K Laboratories, USA; Toxogonin® (obidoxime) (1,1'oxybis(methylene)]bis[4-[(hydroxyimino)methyl]-pyridinium]-dichloride) (fig. 1) from Merck, Darmstadt, Germany; and atropine sulphate from Norsk Medisinal Depot, Oslo, Norway. HI-6 ([[[(4-aminocarbonyl)pyridinio]methoxy]-methyl]-2[(hydroxyimino)methyl]-pyridinium-dichloride (fig. 1) was a gift from Dr Boulet, Defence Research Establishment Suffield, Ralston, Alberta, Canada. Soman (O-[1,2,2-trimethylpropyl]-methylphosphonofluoridate), assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory. All other chemicals were of analytical laboratory grade.

Experimental protocol

In vivo exposure to soman: All animals received TOCP (100 mg/kg, sc) dissolved in 1,2-propanediol 24 hrs before soman exposure in order to inhibit carboxylesterase, an important soman scavenger in rodents (Sterri *et al.* 1981). Pyridostigmine prophylaxis was administered through osmotic pumps (Alzet, model 2001) 18 hrs before soman exposure. The osmotic pumps, which deliver 10 µl/hr, were placed under the skin in the neck region on rats anaesthetized with a fluanisone/fentanyl/midazolam solution (7.10/0.15/3.75 mg/kg, sc). Two rats were exposed simultaneously to soman (3 mg/m^3 for 10 min) in an inhalation chamber designed for exposure to highly toxic gases (see inhalation method). The rats were decapitated immediately after exposure to soman; the trachea and lung were removed, and a blood sample was collected. The trachea from one animal was used to determine of the *ex vivo* nerve-mediated contraction response (see Physiological method). The trachea from the other animal, and lung and plasma from both animals were prepared and used for biochemical determination of ChE activity (see Biochemical methods). The physiological response and ChE activity were followed for at least 90 min from decapitation. The homogenates from which the ChE activity was determined, were incubated at 37°C.

In the experiments with HI-6 or Toxogonin® treatment the rats received TOCP, but not pyridostigmine, 24 hrs before soman inhalation (2.3 ± 0.2 mg/m^3 for 10 min). The oximes were injected immediately after the inhalation exposure to soman. The animals were decapitated 15 min after oxime injection, thereafter treated in the same way as in the pyridostigmine experiments. In addition, some experiments were performed with both pyridostigmine pretreatment and oxime treatment (a combination of the two protocols described above).

In vitro exposure to soman: Rats without any pretreatment were decapitated and their tracheas were removed and used to determine the *in vitro* nerve-mediated contraction response after addition of soman and HI-6 or Toxogonin®. Addition of soman (0.1 µmol/l in the organ bath) was followed by 3 washouts after 7 min, and addition of oxime (100 µmol/l in the organ bath) after 10 or 30 min. Following the physiological experiments the rat trachea was frozen (-20°C) and the ChE activity determined within 1 week.

Inhalation method

Whole body exposures to soman were carried out in a dynamic inhalation system designed specifically for the exposure of small rodents to highly toxic gases (Aas *et al.* 1985). Two rats were exposed simultaneously in a glass chamber of 2200 ml. The vapour concentration of soman was measured by passing 100 ml/min of the inhalation chamber atmosphere for 30 min through 10 ml

of isopropanol which was analyzed by gas chromatography on a Carlo Erba, HRGC 5160 with a nitrogen/phosphorus detector.

Physiological method

After decapitation, the trachea was removed, cannulated, connected to a Statham Transducer (P23AC) via the cannula and filled with KrebsHenseleit buffer. The other end of the trachea was secured onto an electrode whereby the trachea and the transducer formed a sealed system. The tube preparation was then suspended in an organ bath containing a physiological buffer (composition in mmol/l: NaCl 118.4, KCl 4.7, NaHCO3 25.0, KH2PO4 1.16, MgSO4 1.19, CaCl2 2.0 and glucose 11.1), equilibrated with 95% O2/5% CO2 for 30 min before and during the experiment (pH 7.4) and maintained at 37°C (Farmer and Coleman 1970). One electrode was placed outside the trachea, and another inside the tracheal lumen for transmural stimulation. Electrical stimulation (30 V, 0.2 ms, 30 Hz, 3 s trains at 50 s intervals) by a Grass S88 Stimulator resulted in a rapid contraction of the tracheal muscle which produced change in intra-luminal pressure that was recorded by a Grass Polygraph (Model 7) fitted with amplifiers (7 P 1A). Twenty min elapsed between decapitation and the first record of contraction. Indomethacin (1 µmol/l) was present in the buffer to inhibit the production of prostaglandins (Walters *et al.* 1984).

Biochemical methods

The trachea and lung tissues were homogenized (5% w/v) in 20 mmol/l sodium-potassium phosphate buffer (pH = 7.4) by a Polytron instrument (setting 10, 10 sec, ice-cold). The plasma was separated from blood cells by centrifugation (3000 rpm for 10 min) and diluted (1:20) in phosphate buffer before the assay of enzyme activity. For determination of ChE activity after inhibition with pyridostigmine alone, the enzyme preparations were kept ice-cold and measured as quickly as possible (i.e. not more than 25 min after decapitation). The enzyme preparations that were sampled after soman exposure were, on the other hand, incubated at 37°C until and between each measurement of enzyme activity (i.e. kept at the same temperature as the trachea on which the cholinergic response was monitored). ChE activity was determined by the radiochemical method of Sterri and Fonnum (1978): 10 µl of the enzyme solution was mixed with 10 µl of substrate solution which consisted of 2.6 mmol/l [^{14}C]ACh (0.49 Ci/mol) and 20 mmol/l phosphate buffer, pH 7.4. The mixture was incubated in a microtube at 30°C for 15 min, and then transferred to a scintillation vial where the reaction was stopped by dilution with 1 ml of 0.1 mol/l phosphate buffer, pH 7.4. After addition of 0.2 mol/l trioctylammonium phosphate in isoamylalcohol and 10 ml of Insta-Fluor scintillation mixture, the labelled acetate was extracted into organic phase by shaking the mixture lightly for 1 min and placed in the scintillation counter. AChE activity was measured after inhibition of butyrylcholinesterase (BuChE) activity with ethopropazine (Todrik 1954). Protein content was determined by the method of Lowry *et al.* (1951).

Statistics

Data are given as means + SEM. The data were analyzed by analysis-of-variance with repeated measurements techniques. The factors used were the group factor and the time factor.

RESULTS

The transmural stimulation of the intact trachea induced a rapid and reproducible contraction of the tracheal smooth muscle, measured as an increase in intraluminal pressure (fig. 2A). The contraction response was completely blocked by atropine (1 µmol/l) and tetrodotoxin (3 µmol/l) (not shown). Addition of soman in cumulative concentrations induced a concentration dependent response pattern, with increasing response at low concentrations of soman followed by decreasing

response due to a contraction (rise of baseline) at higher concentrations (fig. 2B). Inhalation of soman (2.3 + 0.2 mg/m^3 for 10 min) immediately before decapitation abolished the nerve-mediated tracheal contraction response almost completely (fig. 2C).

Carbamate prophylaxis

Continuous administration of pyridostigmine by the subcutaneous osmotic pump inhibited the ChE activity in trachea, lung and plasma in a dose-dependent manner (tab. 1). The lowest dose (0.01 mg/hr), which inhibited the tracheal ChE activity by 30%, was chosen as a pretreatment for the experiments with inhalation of soman. Pyridostigmine pretreatment reduced the effect of soman inhalation on the cholinergic contraction response. Soman significantly reduced the response by 85%. Pretreatment with pyridostigmine decreased the reduction to 40%, not significantly different from control (figs. 2D and 3). The physiolcgical response in the pyridostigmine pretreated animals increased during the first 60 min after decapitation. The ChE activity in plasma, but not in trachea and lung, was significantly higher in pretreated compared to untreated animals (fig. 4). The mean ChE activities in trachea and lung were, however, elevated to the same degree, and the lack of significance was due to a larger individual variation of the ChE activities in these tissues. The activity did not increase in any of the assayed tissues during the period of measurement.

Treatment with oximes

Oximes (HI-6 or Toxogonin®, 50 mg/kg ip) were given immediately after soman inhalation, and the rats were decapitated 15 min after injection of oxime. A substantial recovery of the physiological response was achieved by HI-6 (figs. 2E and 5) and the contraction response was not significantly different from control. It was, however, possible to observe a difference in the form of the single contractions from the tracings. There is a minor increase in the width at the basis of each contraction in the exposed rats (fig. 2E) compared to the control rats (fig. 2A). The physiological response after treatment with HI-6 increased the first 50 min after decapitation (fig. 5). Toxogonin® was not as effective as HI-6, although the initial responses (at 20 min) were not significantly different from each others (figs. 2F and 5). The recovery achieved by treatment with Toxogonin® was, however, not stable, and the physiological response 30 min after decapitation was not significantly different from the response induced in the animals without any treatment at all after the soman inhalation (fig. 5). Toxogonin® did not reactivate the ChE activity at all, and the small reactivation with HI-6 was not significantly better than Toxogonin® except in plasma at one time point (78 min).

Combination of carbamate prophylaxis and oxime treatment

The combination of pyridostigmine prophylaxis and HI-6 treatment after soman inhalation produced a significant initial recovery of the physiological response, while HI-6 alone did not (figs. 2G and 5). The overall cholinergic contraction response (20-90 min) was, however, not significantly different neither from control nor from that produced by treatment with HI-6 alone. The reactivation of the tracheal ChE activity after pyridostigmine/HI-6 therapy was significantly different from untreated animals at 78 and 138 min only (fig. 6A). The combination therapy produced no reactivation of the ChE activities in lung and plasma (figs. 6B and C). Pyridostigmine pretreatment used in combination with Toxogonin® after soman inhalation resulted in contraction responses that were not significantly different from control (figs. 2H and 5). The combination of pyridostigmine and Toxogonin® was, however, not as potent as HI-6 in restoring the contraction response measured 60 min after decapitation. Furthermore, the form of the electrically induced contractions were closer to control after treatment with HI-6 alone than after pyridostigmine/Toxogonin® treatment. There was, in agreement with the physiological results, a more potent reactivation of the ChE activity in the trachea after pyridostigmine/Toxogonin®

therapy compared to when Toxogonin® was used alone (fig. 6A). The pyridostigmine/Toxogonin® combination therapy did, as the pyrido-stigmine/HI-6 combination, not produce any significant reactivation of the ChE activities in lung and plasma (figs. 6B and C).

Effect of oximes on *in vitro* exposure to soman

Both oximes (100 µmol/l) produced an immediate and significant recovery of the contraction response when added 10 min after soman (0.1 µmol/l) (figs. 2I, 2J and 7). After 10 min oxime effects exceeded those of the controls by more than 100 percent, and decreased thereafter. The recovery induced by Toxogonin® was, in accordance with the results from the *in vivo* exposure, less stable, and at 90 min after addition of oxime only about 50% of controls and not significantly different from soman-exposed controls (fig. 7). The effect of HI-6 was, however, more stable and 90 min after addition still significantly different from soman-exposed controls. A quite different picture appeared when the oximes were added 30 min after addition of soman. HI-6 was almost without effect, and the recovery produced by Toxogonin® was only transient (figs. 2K, 2L and 7). We were not able to measure any reactivation of the tracheal ChE activity in the *in vitro* experiments, regardless of type of oxime used and time of addition of oximes (not shown).

DISCUSSION

The transmurally induced contractions were atropine and tetrodotoxin sensitive, indicating that the contractions were due to stimulation of vagal cholinergic nerves. Soman induced, dependent on the concentration, two different effects on the nerve-mediated contraction response. The enhancement of the muscle contractions at low concentrations of soman was probably due to retarded but complete hydrolysis of the released ACh between subsequent transmural stimulations. The reduction of the transmurally stimulated contraction after addition of higher concentrations of soman was due to a raise in the baseline tension, indicating incomplete hydrolysis of ACh between subsequent stimulations.

Pretreatment with pyridostigmine protected the cholinergic response in the rat trachea after inhalation of soman. This protection was probably due to reversible carbamylation of the AChE, which temporarily renders the enzyme insensitive to irreversible inhibition by soman. The pyridostigmine prophylaxis inhibited the tracheal ChE activity by 30%, and it has been shown that carbamate prophylaxis provides some protection against soman toxicity at levels of inhibition as low as 10% (Lennox *et al.* 1985). It is also possible that the protection afforded by carbamate prophylaxis to some extent may be due to down-regulation of muscarinic receptors. Reduction in the density of muscarinic receptors has been shown in the striata of guinea pigs after continuous infusion of another carbamate, physostigmine (Lim *et al.* 1988), and in the rat airways after subacute inhalation of soman (Aas *et al.* 1987). The contraction response increased dramatically the first 40 min of measurement (i.e. 20-60 min after decapitation) in the animals pretreated with pyridostigmine before soman inhalation, indicating that we may measure the spontaneous decarbamylation and thereby reactivation of the physiologically most important AChE.

There was, however, a considerable discrepancy between the reactivation pattern of ChE activity and the recovery of physiological response. We were not able to detect any increase in ChE activity in the trachea, lung or plasma from the pyridostigmine pretreated animals that could correlate to the increase in elicited contractions during the first 40 min of measurement of physiological response. This may be due to the fact that the physiologically important ChE is a small part of the total ChE. The ChE of intact tissue has been classified into an internal and

external pool on the basis of the effect of drugs with different ability to cross membranes (Burgen and Hobbiger 1951; McIsaac and Koelle 1959; Mittag *et al.* 1971). It has been suggested that the external ChE measured in intact tissues (eye and diaphragm) is a better correlate to the physiological function than the ChE in homogenates, which would represent a mixture of the internal and external ChE (Mittag *et al.* 1971; Lund Karlsen and Fonnum 1977). The discrepancy in our results between physiological effect and ChE activity may be due to the fact that soman readily cross membranes and therefore inhibits both pools of ChE, while pyridostigmine and the oximes, on the other hand, only protect and reactivate the external pool. Results from experiments on the skeletal muscle of the frog has, however, shown that the ChE activity measured in homogenates from this tissue presented a better picture of the *in situ* ChE activity than did the external activity measured in whole muscle (Miledi *et al.* 1984). Which assay method of ChE activity that is most relevant to physiological function may therefore vary between different tissues. The results from our study indicates that ChE activity in homogenates from airway tissue poorly correlates with the physiologically important ChE.

The recovery of the cholinergic response provided by treatment with HI-6 immediately after exposure to soman, show that HI-6 is very effective in rat under these conditions. The time lapse between poisoning and therapy has been shown to be important for the effectiveness of oxime therapy against organophosphorus poisoning (Harris *et al.* 1969). This is in accordance with our results from the *in vitro* exposure experiments with the isolated tracheal preparation, where addition of HI-6 was effective 10 min but not 30 min after addition of soman. The ineffectiveness of the oximes 30 min after addition of soman is probably due to the formation of irreversibly inhibited (aged) enzyme. The soman-inhibited ChE from most species ages, and thereby becomes resistant to reactivation, very rapidly (within 6 min) both *in vivo* and *in vitro* (Fleisher and Harris 1965; Berry and Davies 1966; Fleisher *et al.* 1967). The process of aging is, in accordance with our results, probably somewhat slower in rodents (Wolthuis and Kepner 1978). Toxogonin® produced a better recovery of the contraction response than HI-6 when added 30 min after addition of soman, indicating that effects other than enzyme reactivation may be present. The effect of Toxogonin® was, however, only transient.

The protection provided by treatment with Toxogonin® after soman inhalation was not stable. The recovery induced by addition of Toxogonin® 10 min after *in vitro* exposure to soman was substantial, but in accordance with the *in vivo* treatment, not stable. HI-6 therefore seems to provide a more stable recovery of the cholinergic response. The *ex vivo* ChE activities were identical in Toxogonin® treated and untreated animals, and the small reactivation produced by HI-6 was not statistically significant. However, the results from the *in vitro* experiments, showing that HI-6 is effective 10 min after soman, but completely without effect 30 min after soman, suggest that the main mechanism behind the effect of HI-6 is reactivation of ChE that has not yet aged. We were not able to measure any reactivation of ChE in the *in vitro* preparations. The reactivation may be small, since only a small part of the total capacity of ChE is probably needed, and could easily disappear due to reinhibition by soman released from the tissues in the time lapse between exposure and assay.

The organophosphate inhibited ChE reacts with oximes and give phosphylated oxime plus reactivated enzyme. The phosphylated oximes have in several instances shown to be very potent inhibitors of ChE (Hackley *et al.* 1959; Rogne 1967; Barstad *et al.* 1969; Schoene 1972), but are usually relatively unstable and decompose to oxime and alkylphosphonic acid (Fonnum 1975). Complexes between pyridinium-2-oximes and organophosphates are too unstable to be studied in detail, and it is the pyridinium-4-oximes that has been shown to give the most stable phosphylated oximes (Hackley *et al.* 1959; De Jong and Ceulen 1978). Toxogonin®, in contrast to

HI-6, belongs to the latter group, and the decrease in cholinergic contraction response *ex vivo* and *in vitro* may be due to reinhibition of the AChE by phosphylated oxime.

Combination of pyridostigmine prophylaxis and HI-6 treatment provided a better protection of the initial physiological response. The response 60 min after decapitation was, however, not significantly different from control or treatment with HI-6 alone. A more additive therapeutic effect of pyridostigmine prophylaxis and HI-6 treatment would perhaps be expected if the animals had been more heavily intoxicated. The combination of pyridostigmine and Toxogonin® provided a more effective initial protection of the physiological response than pyridostigmine alone, and the response was more stable than after treatment with Toxogonin® alone. The reactivation of the ChE activity was primarily enhanced in the trachea by the combination therapy.

The results of this study indicate that HI-6 is more potent than Toxogonin® in providing a stable recovery of an *ex vivo* peripheral cholinergic response in the rat after 10 min inhalation exposure to soman. The time dependence of the *in vitro* effect of HI-6 suggest that the main mechanism is reactivation of ChE.

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LEGENDS TO FIGURES

Fig.1. Structures of HI-6 and Toxogonin®.

Fig.2. Representative tracings showing tracheal muscle contractions elicited by electrical field stimulation: (A) control; (B) cumulative application of soman (final concentrations shown); (animals C-H were subjected to inhalation of soman (1.4-3 mg/m^3) for 10 min before decapitation) (C) without any treatment; (D) pyridostigmine (0.01 mg/hr) prophylaxis for 18 hrs prior to exposure to soman; (E) HI-6 (50 mg/kg, ip) immediately after exposure to soman and 15 min before decapitation; (F) Toxogonin® (50 mg/kg, ip) immediately after exposure to soman and 15 min before decapitation; (G) pyridostigmine prophylaxis and HI-6 treatment (D + E); (H) pyridostigmine prophylaxis and Toxogonin® treatment (D + F); (I-L are tracings from experiments with *in vitro* exposure to soman (0.1 µmol/l)) (I) addition of HI-6 (100 µmol/l) 10 min after addition of soman (0.1 µmol/l); (J) addition of Toxogonin® (100 µmol/l) 10 min after addition of soman; (K) addition of HI-6 (100 µmol/l) 30 min after addition of soman; and (L) addition of Toxogonin® (100 µmol/l) 30 min after addition of soman.

Fig.3. Effect of continuous pretreatment with pyridostigmine (0.01 mg/hr administered subcutaneously by a mini-osmotic pump) for 18 hrs on the tracheal contraction elicited by electrical field stimulation after inhalation exposure to soman (3 mg/m^3 for 10 min). (●) control; (▲) pyridostigmine prophylaxis before soman inhalation; (Δ) soman inhalation without any treatment (0.9% saline solution). Means ± SEM from 5-7 animals.

Fig.4. Effect of continuous pretreatment with pyridostigmine (0.01 mg/hr) on the cholinesterase activity (nmol ACh hydrolysed/min/mg protein) in: (A) trachea; (B) lung; and (C) plasma from rat after inhalation of soman (3 mg/min) for 10 min. (●) control; (▲) pyridostigmine prophylaxis for 18 hrs before soman inhalation; and (Δ) soman inhalation without any treatment. The cholinesterase activity was measured as described in Methods. Means ± SEM from 5-7 animals.

Fig.5. Effect of different treatments on the electrically elicited tracheal smooth muscle response after inhalation exposure to soman (1.4-2 mg/m^3 for 10 min). (●) control; (Δ) HI-6 (50 mg/kg ip) immediately after exposure to soman and 15 min before decapitation; (▽) Toxogonin® (50 mg/kg, ip) immediately after exposure to soman and 15 min before decapitation; (▲) pyridostigmine prophylaxis (0.01 mg/hr) for 18 hrs prior to exposure to soman, and HI-6 (50 mg/kg, ip) immediately after exposure to soman and 15 min before decapitation; (▼) pyridostigmine prophylaxis (0.01 mg/hr) for 18 hrs prior to exposure to soman, and Toxogonin® (50 mg/kg, ip) immediately after exposure to soman and 15 min before decapitation; and (O) exposure to soman without any treatment. Means ± SEM from 6-8 animals.

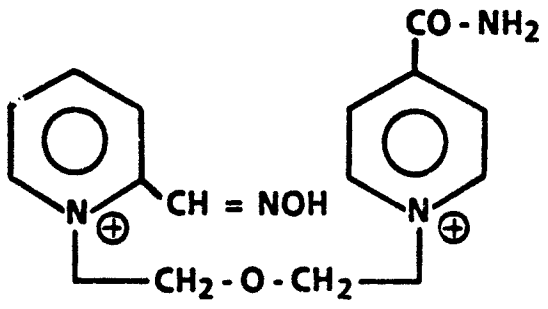
Fig.6. Effect of different treatments on the cholinesterase activity (nmol ACh hydrolysed/min/mg protein) in: (A) trachea; (B) lung; and (C) plasma from rat after inhalation exposure to soman (1.4-2 mg/m^3 for 10 min). (●) control; (Δ) HI-6 (50 mg/kg ip) immediately after exposure to soman and 15 min before decapitation; (▽) Toxogonin® (50 mg/kg ip) immediately after exposure to soman and 15 min before decapitation; (▲) pyridostigmine prophylaxis (0.01 mg/hr) for 18 hrs prior to exposure to soman, and HI-6 (50 mg/kg ip) immediately after exposure to soman and 15 min before decapitation; (▼) pyridostigmine prophylaxis (0.01 mg/hr) for 18 hrs prior to exposure to soman, and Toxogonin® (50 mg/kg ip) immediately after exposure to soman and 15 min before decapitation; and (O) exposure to soman without any treatment. Means ± SEM from 6-8 animals.

Fig.7. Effect of addition of soman and oximes on the tracheal contraction response elicited by electrical stimulation. Soman (0.1 µmol/l) was added at time 0, and HI-6 or Toxogonin® (100 µM) were added 10 or 30 min after *in vitro* addition of soman. (Δ) control; (▲) HI-6 10 min after soman; (▼) Toxogonin® 10 min after soman; (○) HI-6 30 min after soman; (●) Toxogonin® 30 min after soman; and (▼) no addition of oxime after soman. The tracheal contraction in absolute units (mmH_2O) before addition of soman and oxime, are represented as columns (insert, top right). Means ± SEM from 6-10 experiments.

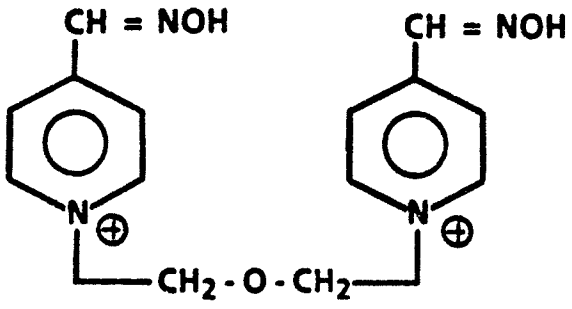
TABLE 1. The effect of continuous subcutaneous administration of different doses of pyridostigmine on the cholinesterase activity in different tissues.

Pyridostigmine (mg/hr)	n	Percent inhibition of ChE activity		
		Plasma	Lung	Trachea
0.01	6	44 ± 3	25 ± 8	30 ± 7
0.02	4	53 ± 6	41 ± 6	50 ± 1
0.04	3	63 ± 5	46 ± 2	52 ± 5

Pyridostigmine was administered and cholinesterase activity was measured as described in Methods. The tissues were kept ice-cold during preparation, and the cholinesterase activity was assayed within 25 min from decapitation. Means ± SEM from n animals.



HI - 6



TOXOGONIN

Figure 1

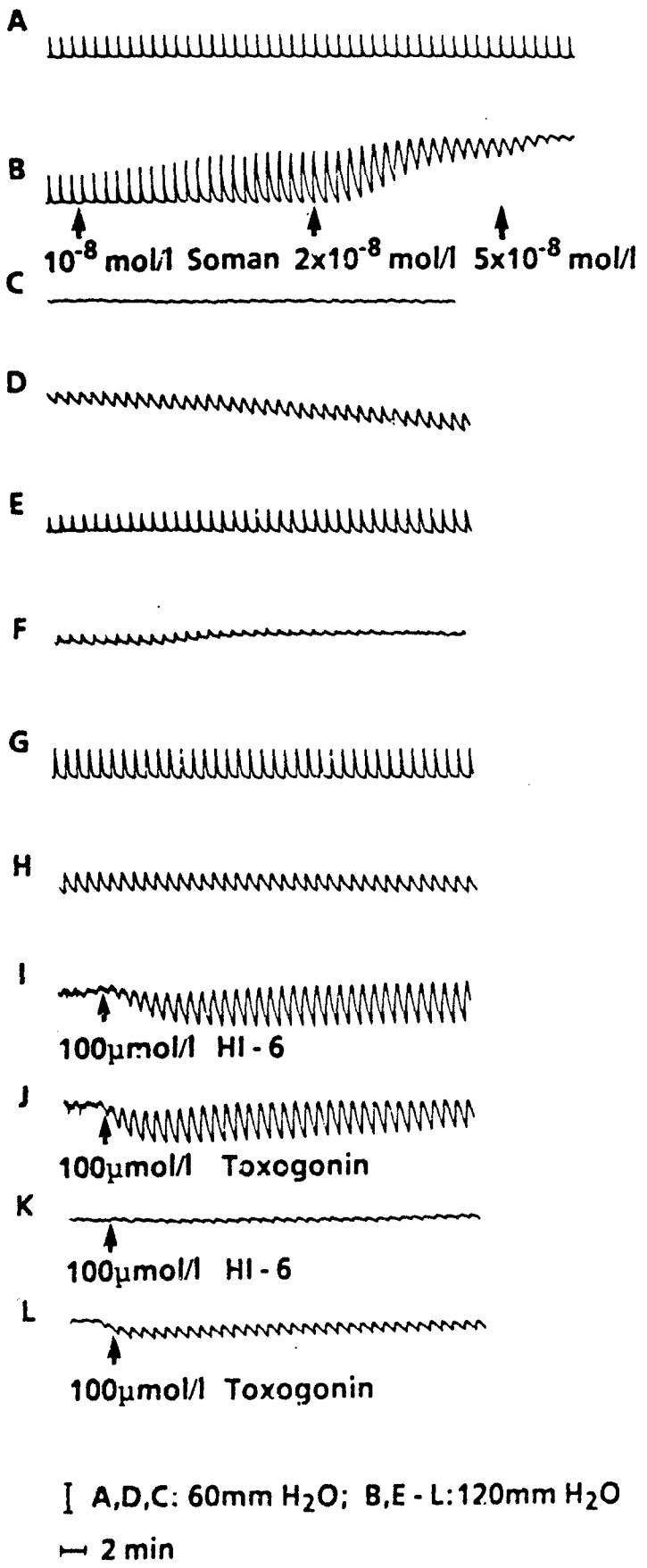

A
B
10^{-8} mol/l Soman
$2x10^{-8}$ mol/l
$5x10^{-8}$ mol/l
C
D
E
F
G
H
I
100µmol/l HI-6
J
100µmol/l Toxogonin
K
100µmol/l HI-6
L
100µmol/l Toxogonin
A,D,C: 60mm H_2O; B,E-L: 120mm H_2O
2 min

Figure 2

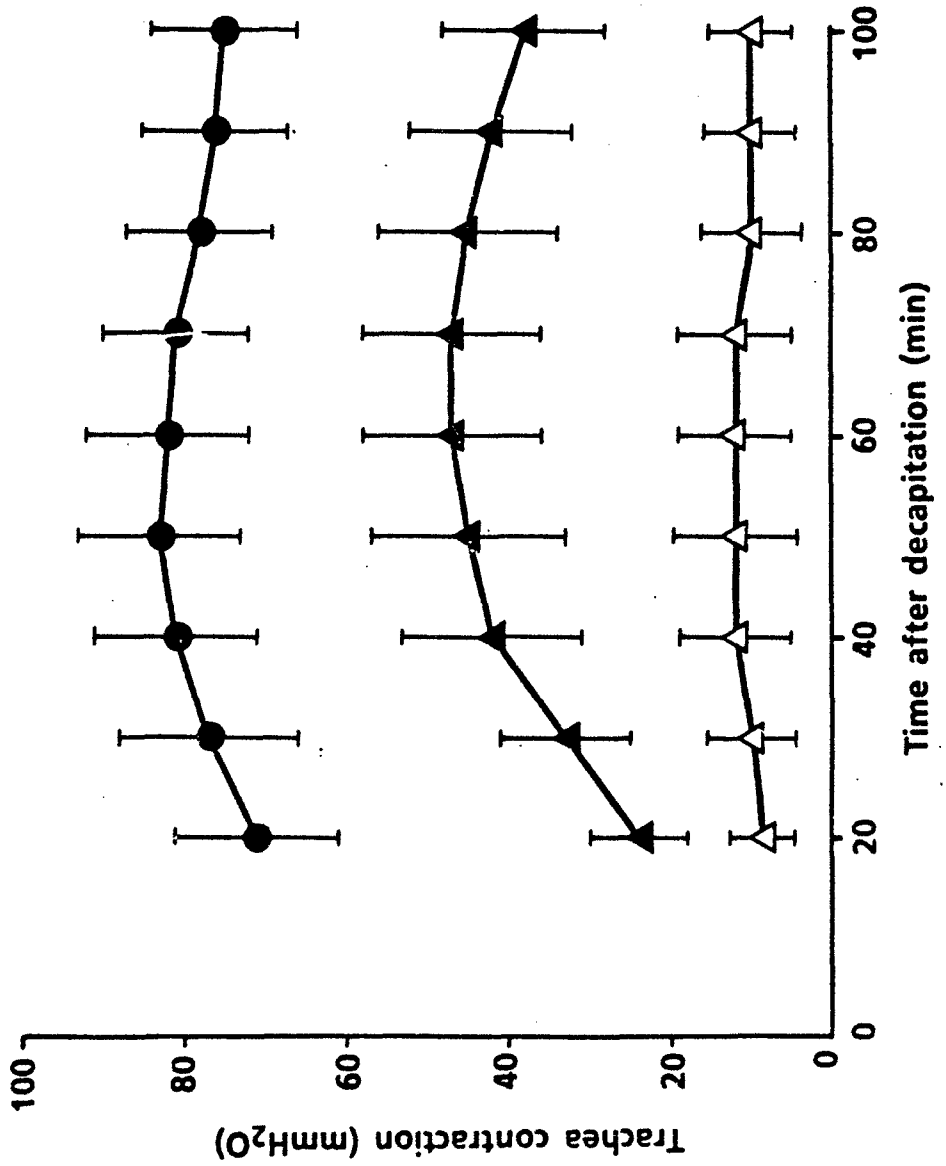

Trachea contraction (mmH2O)
100
80
60
40
20
0
0
20
40
60
80
100
Time after decapitation (min)

Figure 3

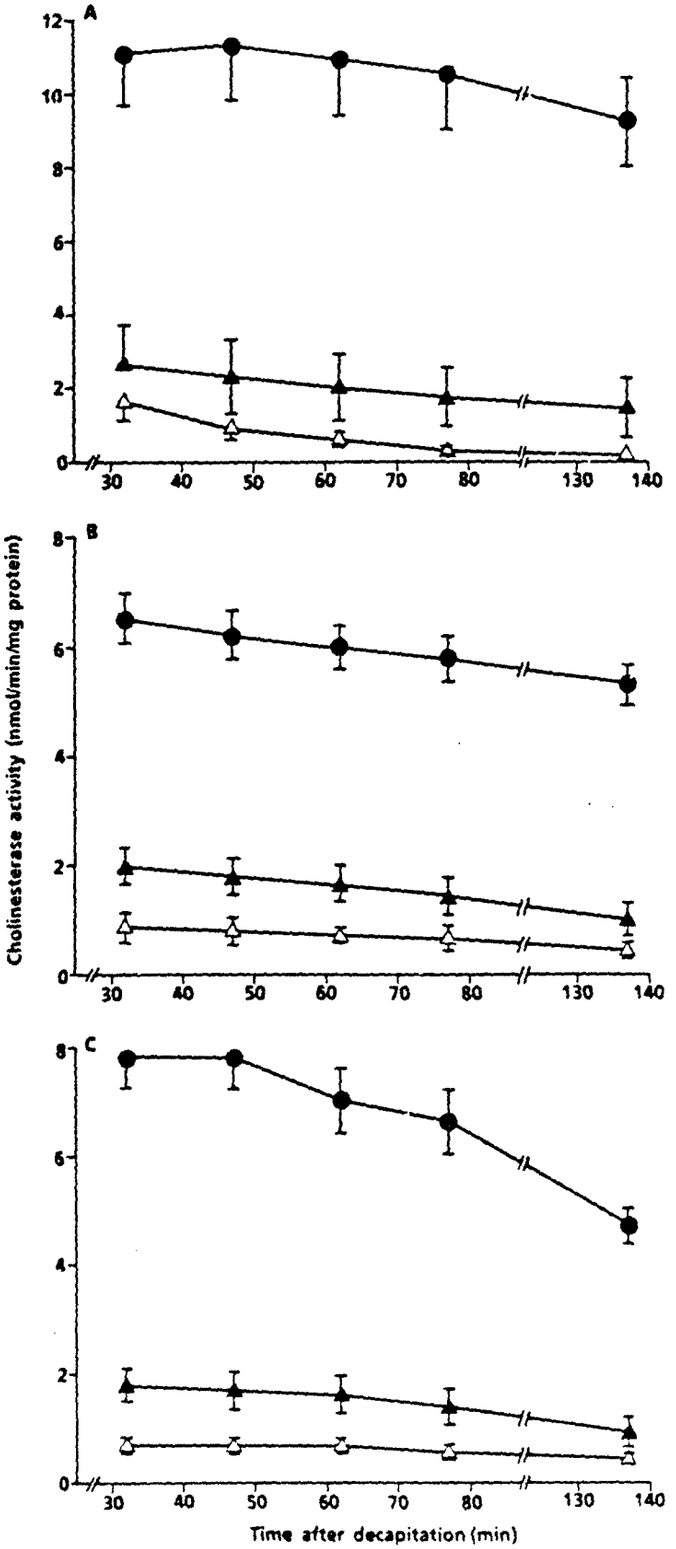

A
B
C
Cholinesterase activity (nmol/min/mg protein)
Time after decapitation (min)
30
40
50
60
70
80
130
140

Figure 4

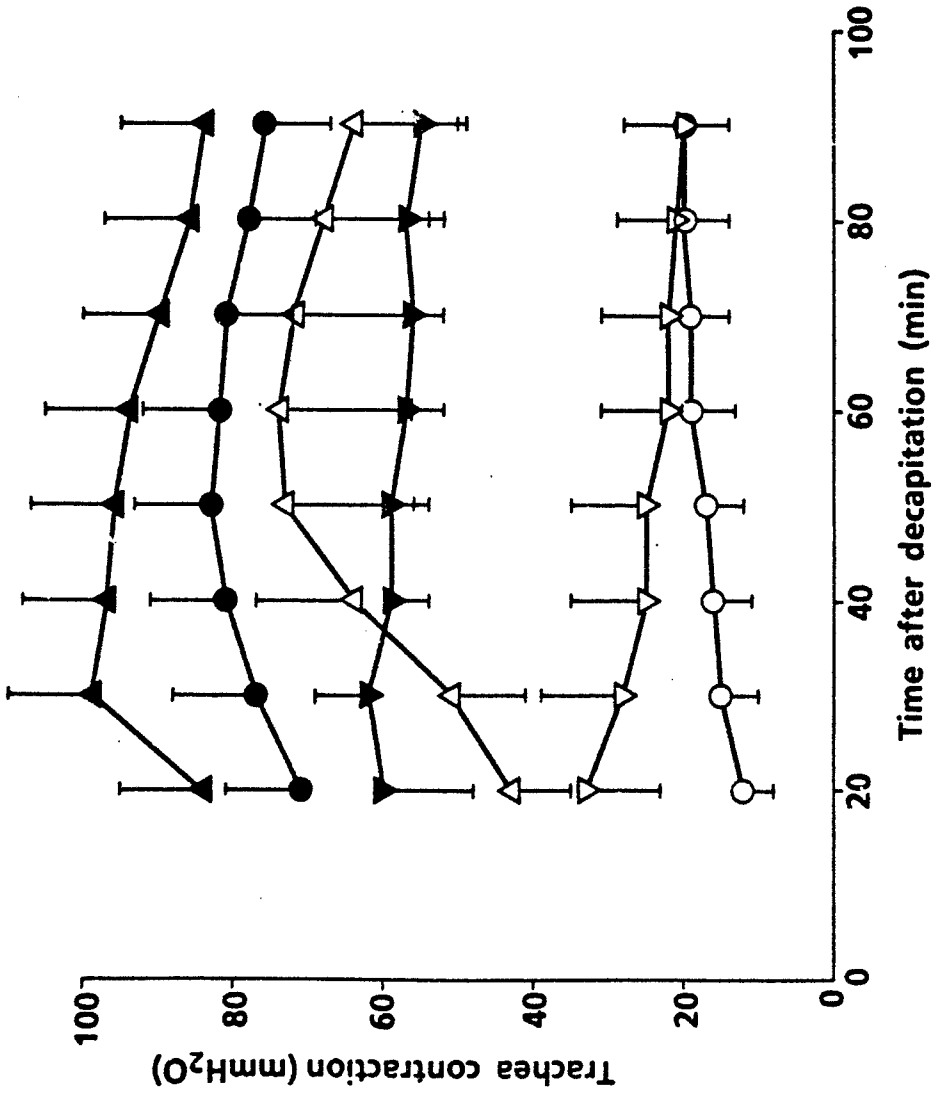

Trachea contraction (mmH2O)
100
80
60
40
20
0
0
20
40
60
80
100
Time after decapitation (min)

Figure 5

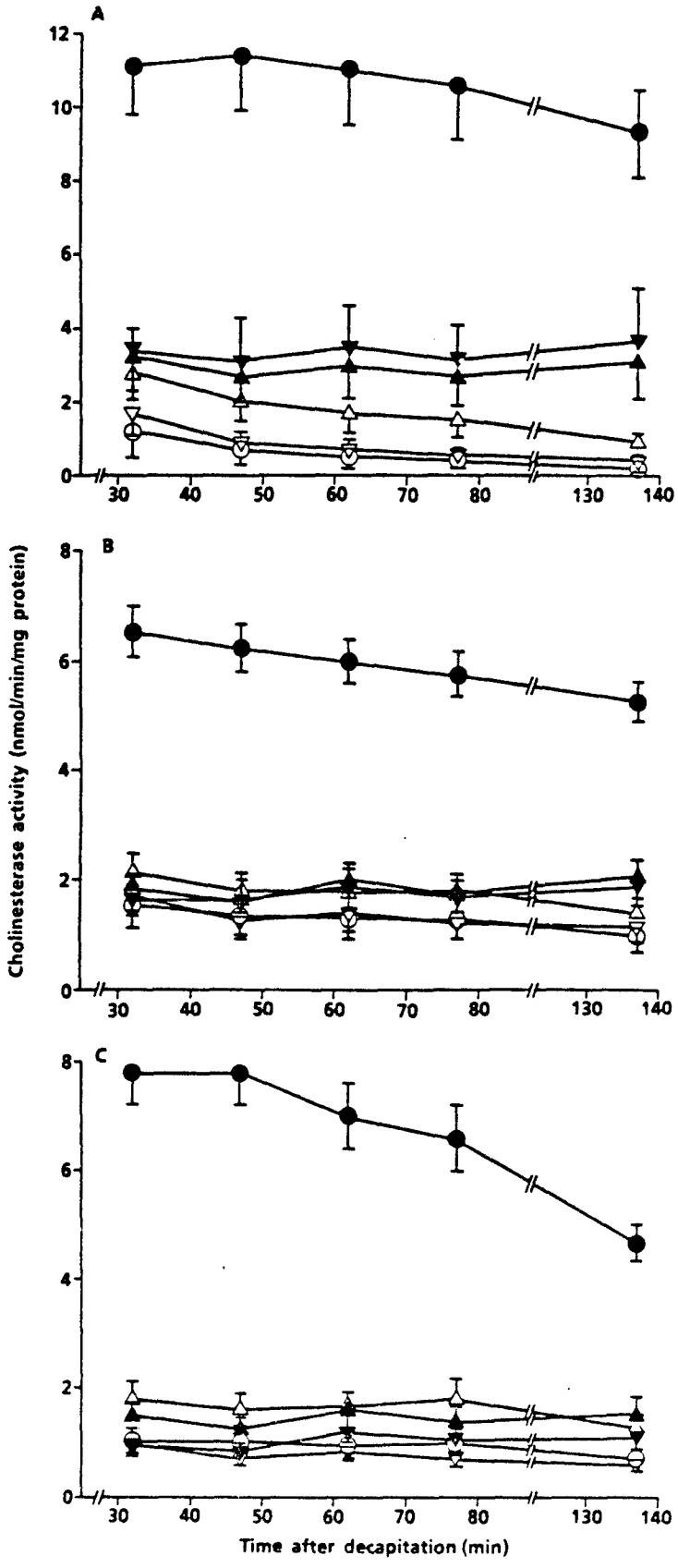

A
B
C
12
10
8
6
4
2
0
30
40
50
60
70
80
130
140
Cholinesterase activity (nmol/min/mg protein)
Time after decapitation (min)

Figure 6

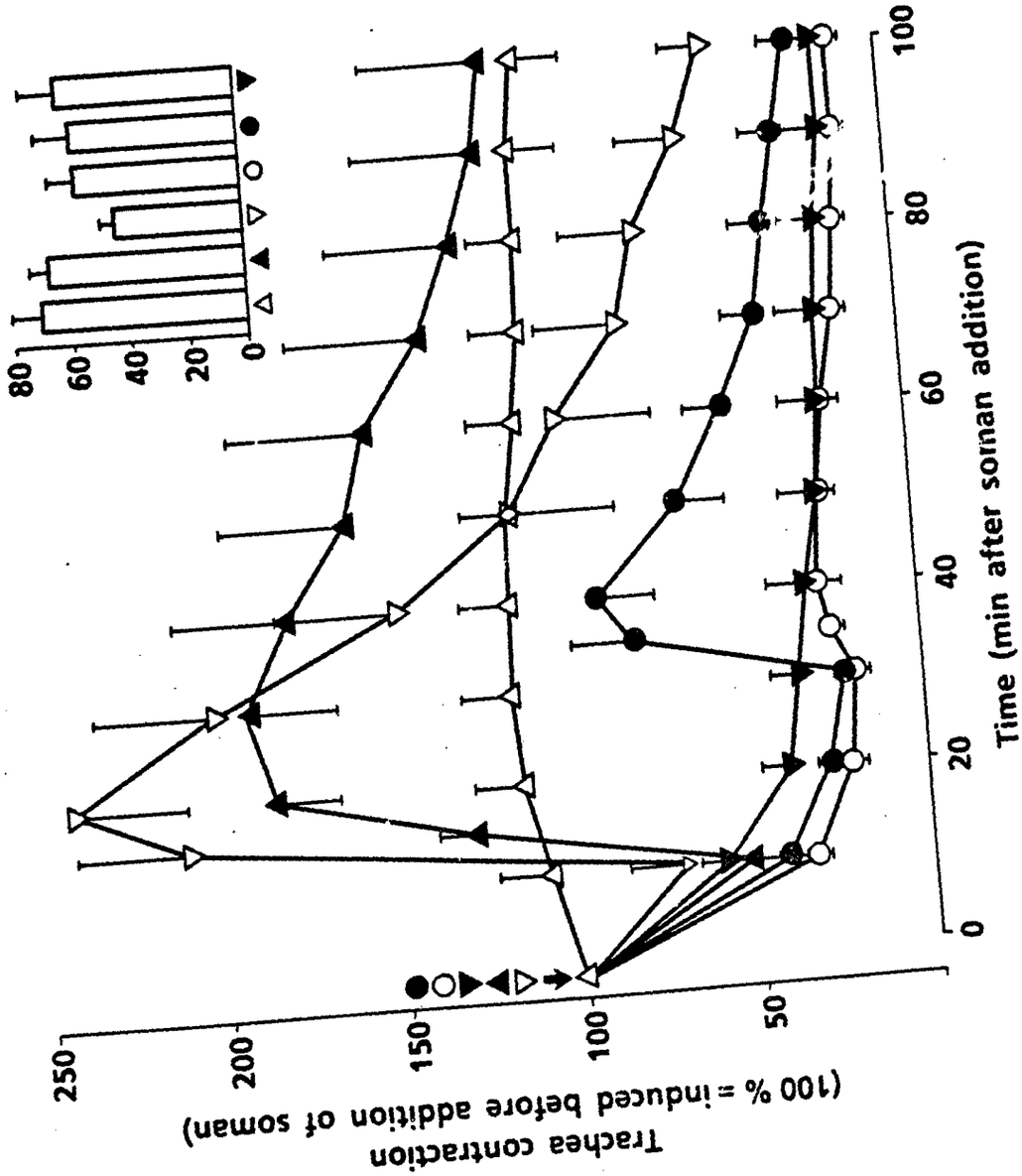

Trachea contraction
(100 % = induced before addition of soman)
250
200
150
100
50
0
20
40
60
80
100
Time (min after soman addition)
80
60
40
20
0

Figure 7

Paper III

EFFECT OF CALCIUM ANTAGONISTS (ω-CONOTOXIN GVIA, VERAPAMIL, GALLOPAMIL, DILTIAZEM) ON BRONCHIAL SMOOTH MUSCLE CONTRACTIONS INDUCED BY SOMAN.

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SUMMARY.

The effect of the calcium antagonists ω-conotoxin GVIA, verapamil, gallopamil and diltiazem was investigated on *in vitro* bronchial smooth muscle contraction in the rat induced by the nerve agent soman. Soman inhibits the acetylcholinesterase activity irreversibly. The effect of the calcium channel antagonists on contractions induced by electrical field stimulation and carbachol was also investigated, in order to elucidate the mechanism by which calcium antagonists inhibit the soman induced contraction.

ω-Conotoxin GVIA reduced the bronchial smooth muscle contraction induced by electrical field stimulation with an almost complete inhibition at approximately $1.0x10^{-6}$ M. The soman induced contraction was only inhibited by 15 percent at a concentration of $3.0x10^{-6}$ M ω-conotoxin GVIA. The organic calcium antagonists verapamil, gallopamil and diltiazem reduced both electrically and soman induced smooth muscle contraction. Complete inhibition of the contractions induced by soman was achieved at $1.4x10^{-4}$ M for verapamil and gallopamil, while diltiazem inhibited the contraction to 7 percent of control at $1.4x10^{-4}$ M. Verapamil, gallopamil and diltiazem increased the EC_{50} for carbachol significantly, while ω-conotoxin GVIA had no effect. None of the calcium antagonists had any effect on the maximal contraction induced by carbachol. Verapamil, gallopamil and diltiazem blocked, however, sub-maximal contractions induced by carbachol (10^{-7}-10^{-5} M) resulting in a right-shift of the dose response curve.

The results show that ω-conotoxin GVIA inhibits the calcium-dependent release of acetylcholine which causes contraction of airway smooth muscle, while it has no effect on smooth muscle contraction induced by soman. Gallopamil, verapamil and diltiazem inhibit the contraction of bronchial smooth muscle following stimulation of postjunctional muscarinic receptors resulting from inhibition of the acetylcholinesterase activity by soman. Gallopamil and verapamil inhibit the contraction more potently than diltiazem.

Airway smooth muscle - vagus nerve - acetylcholine - calcium antagonists

INTRODUCTION

Organophosphorus compounds are extensively used as insecticides in agriculture, and some are potential warfare agents. The acute toxic effect of organophosphorus compounds is due to their inhibition of acetylcholinesterase activity, and the subsequent accumulation of extracellular acetylcholine at the cholinergic terminals. The primary route of uptake is, for some organophosphates, by inhalation, and the symptoms after acute intoxication include bronchoconstriction, increased bronchial secretion and weakness or paralysis of respiratory muscles. The conventional

treatment of organophosphate intoxication includes atropine and oxime administration, although reactivation of acetylcholinesterase activity by oxime treatment has proven difficult after inhibition with the organophosphate soman (Heilbronn and Tolagen 1965; Clement 1979). Previous studies have shown that soman has a substantial effect on the smooth muscle response and the acetylcholinesterase and carboxylesterase activities in rat airways (Aas *et al.* 1986, 1987; Walday *et al.* 1991) as well as in guinea pig bronchial smooth muscle (Aas *et al.* 1988).

Calcium plays an important role as an intracellular messenger to trigger both the release of neurotransmitters (Katz and Miledi 1965) and the contraction of muscles (Ebashi and Endo 1968). Electrophysiological techniques have shown the existence of three different groups of voltage-gated calcium channels, L-, N- and T-types (Nowycky *et al.* 1985; Fox *et al.* 1987; Tsien *et al.* 1988).

ω-Conotoxin GVIA is a 27 amino acid peptide isolated from the venom of a marine mollusc, *Conus geographus* (Olivera *et al.* 1984). ω-Conotoxin GVIA appears to bind to the N-type voltage-sensitive calcium channel and thereby reduce the depolarizing-induced influx of calcium required for neurotransmitter release (Kerr and Yoshikami 1984; Cruz *et al.* 1987). ω-Conotoxin GVIA has also been shown to block L-type calcium channels (Miller 1987). Only the L-type calcium channels are blocked by phenylalkylamines (verapamil and gallopamil), benzothiazepines (diltiazem) and dihydropyridines (nifedipine). T-type channels, on the other hand, are relatively insensitive to the organic and to most inorganic calcium antagonists (see Nayler 1988).

Earlier studies suggest that ω-conotoxin GVIA has little effect on mouse nerve-muscle preparations (Olivera *et al.* 1985; Sano *et al.* 1987; Anderson and Harvey 1987). Recent reports provide, however, evidence for ω-conotoxin GVIA sensitive N-type calcium channels in several mammalian autonomic peripheral nerve muscle junctions such as myenteric plexus, blood vessels, heart, vas deferens, taenia caeci, colon, and urinary bladder (Maggi *et al.* 1988; Hirning *et al.* 1988; Lundy and Frew 1988; Wessler *et al.* 1990; Pruneau and Angus 1990; DeLuca *et al.* 1990). The effect of ω-conotoxin GVIA has so far not been investigated in airway smooth muscle. The effects of verapamil on airway smooth muscle have been well documented. *In vitro* studies show that verapamil reverses the tracheal smooth muscle contraction in guinea-pigs (Deal *et al.* 1984; Foster *et al.* 1984) and dogs (Walter *et al.* 1982). The *in vivo* effect of verapamil is, however, probably dependent on the specific contraction-inducing stimuli (Lindeman *et al.* 1991).

The object of the present study was to investigate whether calcium antagonists block bronchial smooth muscle contraction evoked by soman. The potential of these antagonists for inhibiting the contraction induced by organophosphorus compounds depends upon their ability to inhibit contractions induced both by evoked and spontaneously released acetylcholine.

METHODS

Animals

Male Wistar rats (200-300 g) (from Møllegaard, Copenhagen, Denmark) were used. The rats were given a standard laboratory diet and given water *ad libitum*. The animals were kept in standard laboratory cages, six in each, for approximately two weeks before the start of the experiments. The sawdust bedding was replaced daily to ensure that the concentration of ammonia was kept at a very low level. The rats had no signs of symptoms of respiratory tract infections. They were killed by decapitation and the bronchi were removed and transferred to the physiological buffer (for composition see Physiological method).

Chemicals

Carbachol, physostigmine salicylate, bovine serum albumin - fraction V, verapamil hydrochloride, methoxyverapamil (gallopamil) and diltiazem hydrochloride were bought from Sigma Chemical Co. (Poole, England), ω-conotoxin GVIA from Research Biochemicals Inc (MA, USA), (1-^{14}C)-acetylcholinechloride from Amersham (England) and atropine sulphate from Norsk Medisinal Depot (Oslo, Norway). Soman (O-[1,2,2-tri-methylpropyl]methyl- phosphonofluoridate), assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory. All other chemicals were of analytical laboratory reagent grade.

Physiological method

The left and right bronchi were mounted in parallel as ring preparations on hooks as previously described by Aas and Helle (1982). The preparations were maintained in a thermostatically controlled organ-bath (50 ml, 37°C) containing Krebs buffer for measurement of isometric smooth muscle contraction. The epithelium was not removed. The buffer had the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; glucose, 11.1 (pH = 7.4). In calcium-free buffer, calcium was substituted by equimolar concentration of magnesium and the preparation was preincubated in this buffer for 30 min before start of experiment. The solutions were gassed with 95% O_2 and 5% CO_2. The drugs were added to the buffer in cumulative concentrations. During electrical field stimulation the bronchi were mounted between platinum electrodes and stimulated (30 Hz and 1.0 ms for 3 s at 40 s intervals, supramaximal voltage) by a Grass S88 stimulator. The preparations were given a preload of 1.0 g tension and equilibrated for 60 min before the start of the experiments. A basal tone of 1.0 g was selected because this gives a maximal response to carbachol that is both rapid and constant (Aas and Helle 1982). The contraction was recorded isometrically by Grass force displacement transducers (FT-13C) and monitored on a Grass polygraph (Model 7) fitted with amplifiers (7 P 1A).

Biochemical methods

The rat bronchi were homogenized (10% w/v) in 20 mM sodium-potassium phosphate buffer (pH = 7.4) (glass/glass homogeniser), 20 strokes, 720 r.p.m., ice-cold) before determination of acetylcholinesterase activity. The acetylcholinesterase activity was measured after inhibition of the butyrylcholinesterase activity with ethopropazine (Todrik 1954). The cholinesterase activities were determined at 30°C by the radiochemical micromethod of Sterri and Fonnum (1978). The protein concentration was determined by the method of Lowry *et al.* (1951).

Statistics

Values given are the means ± SEM or geometric means (IC_{50} and EC_{50}) with n being the number of observations. Results were tested for statistical significance by Student's t-test (Table 3) and Student's t-test for pairwise comparisons (Table 2 and 3), each test having a level of significance equal to 0.05. A modified t-test for multiple comparisons (Table 1) was performed according to Bonferroni (see Wallenstein *et al.* 1980) and the individual p-values were adjusted with respect to the number of comparisons. IC_{50} and EC_{50} values were determined graphically on the basis of the concentration-response curves obtained for ω-conotoxin GVIA, verapamil, gallopamil and diltiazem on the contraction of the bronchial smooth muscle.

RESULTS

Effects of calcium antagonists on electrically and carbachol evoked contractions of bronchial smooth muscle

Electrical field stimulation of rat bronchial smooth muscle in the presence of calcium (2.6 mM) resulted in contractions, which were atropine and tetrodotoxin sensitive (Aas and Helle 1982; Aas and Fonnum 1986). The calcium antagonists ω-conotoxin GVIA, verapamil, gallopamil and diltiazem inhibited in a concentration dependent manner the contraction induced by electrical field stimulation (Fig. 1). ω-Conotoxin GVIA was much more potent than gallopamil and verapamil, while diltiazem was least potent. The IC_{50} values for inhibition of the electrically induced contraction are given in Table 1. An almost complete inhibition of electrical field stimulation induced contractions was observed in calcium-free buffer. The contractions were reduced to 3.8± 1.3 percent of control (n = 7).

Cumulative additions of carbachol (10^{-8}-10^{-4} M) induced a concentration-dependent increase in contraction, which was sensitive to verapamil, gallopamil and diltiazem, but not to ω-conotoxin GVIA (Table 2). There were no effects of the calcium antagonists on the intrinsic activity (α) of carbachol (Table 2).

Sub-maximal contractions (approx. 60%) induced by carbachol ($1.0x10^{-6}$ M), was significantly reduced by verapamil, gallopamil and diltiazem, but not in calcium-free buffer (Table 3). Gallopamil had a substantial effect and reduced the carbachol induced contraction to approximately 7 percent of control, while verapamil and diltiazem had a considerably smaller effect (Table 3).

The effect of omitting calcium from the buffer on contractions induced by physostigmine was also studied since there was a discrepancy between the effects of calcium-free buffer on soman and carbachol induced contractions. There was no significant effect on the physostigmine induced contraction by removal of extracellular calcium (Table 3).

Effects of calcium antagonists on soman induced contractions of bronchial smooth muscle

The calcium antagonists verapamil, gallopamil and diltiazem inhibited in a concentration dependent manner the contraction induced by soman (Fig. 2). Soman was applied in a concentration of $1.4x10^{-7}$ M, which gives a smooth muscle contraction of approximately 50 percent relative to a maximal contraction by carbachol (Table 3). The IC_{50} values for verapamil, gallopamil and diltiazem obtained from the concentration-response curves showed that diltiazem was the least potent ($2.5x10^{-5}$ M), and gallopamil was the most potent antagonist ($2.9x10^{-6}$ M) although not significantly different from verapamil ($3.5x10^{-6}$ M) (Table 1). ω-Conotoxin GVIA, on the other hand, had no significant effect on the soman induced contraction (Fig. 2). Both verapamil and gallopamil completely inhibited the soman induced contraction at $1.4x10^{-4}$ M, while diltiazem reduced the contraction by approximately 90 percent (Table 3). Omitting calcium from the physiological buffer following a soman-induced contraction reduced the smooth muscle response to about the same level as with diltiazem (Table 3). Soman ($1.4x10^{-7}$ M) completely inhibited acetylcholinesterase and butyrylcholinesterase activities in bronchial smooth muscle (Table 4).

DISCUSSION

The contraction of bronchial smooth muscle induced by organophosphorus compounds, such as soman, is primarily due to stimulation of smooth muscle muscarinic receptors both by actively

and spontaneously released acetylcholine that has accumulated following inhibition of acetylcholinesterase. This implies that the potential of calcium antagonists as inhibitors of organophosphate induced contractions, is dependent on either inhibition of both evoked and spontaneous release of acetylcholine from nerve varicosities, or a direct effect on the calcium-channels in smooth muscle. In the present study, we have investigated the effect of four different calcium antagonists on the bronchial smooth muscle contraction induced by (1) endogenous acetylcholine following acetylcholinesterase inhibition by soman, (2) direct muscarinic receptor stimulation with carbachol and (3) transmural nerve stimulation which induce release of acetylcholine.

Contractions induced by electrical field stimulation were inhibited by the calcium antagonists (ω-conotoxin GVIA >> galiopamil > verapamil = diltiazem). The carbachol induced contractions were insensitive to ω-conotoxin GVIA. The effect of ω-conotoxin GVIA on electrical but not carbachol induced contractions indicates that ω-conotoxin GVIA inhibits the evoked release of acetylcholine from the vagal nerve in airway smooth muscle. This is in accordance with previous reports on other tissues, which have shown that ω-conotoxin GVIA primarily block the influx of calcium through calcium channels of the N-type which are thought to supply the calcium necessary in nerve terminals to trigger the release process (Kerr and Yoshikami 1984; Miller 1987; Hirning *et al.* 1988; Lundy *et al.* 1991). This is recently confirmed by the finding that ω-conotoxin GVIA binds exclusively at the active zones on the nerve terminals in the frog neuromuscular junction (Robitaille *et al.* 1990). Furthermore, it has been shown that postjunctional calcium channels in vertebrate cardiac, skeletal, and vascular smooth muscle cells are resistant to ω-conotoxin GVIA (McCleskey *et al.* 1987).

The source of calcium that is recruited to induce contraction following release of acetylcholine seems to depend on the degree of muscarinic stimulation. The organic calcium antagonists inhibit the calcium influx from the extracellular space (Spedding 1982). In our experiments they reduced the sub-maximal contractions induced by low concentrations of carbachol (gallopamil >> verapamil > diltiazem). A part of the recruited calcium seems therefore to originate from the extracellular compartment. The maximal contraction was, however, not affected, which indicates that the recruitment of calcium from intracellular stores is sufficient to induce maximal contraction. This is in accordance with the results obtained by Farley and Miles (1978), who showed that recruitment of calcium from different stores in canine trachea was dependent on the degree of muscarinic stimulation. In these experiments it was shown that the contractions induced by low but not by high concentrations of acetylcholine were sensitive to verapamil. The same mechanism that underlies this difference in recruitment of calcium, may also underlie the difference in peak and steady-state increases in intracellular calcium in swine trachealis which also is dependent on the stimulation intensity (Shieh *et al.* 1991).

The prejunctional leakage of acetylcholine that is supposed to be responsible for soman induced contractions in rat airways, is not dependent on calcium (Aas and Fonnum 1986). The soman induced contractions are inhibited equally or more potently than the nerve-mediated contractions by calcium antagonists, indicating that their primary site of action is postjunctional rather than prejunctional. This is in accordance with previous reports which show that the organic calcium antagonists are without effect on the evoked release of neurotransmitters in a number of different tissues (Kaplita and Triggle 1983; Wessler *et al.* 1990). The lack of effect of ω-conotoxin GVIA on the soman induced contractions is consistent with the presumed prejunctional site of action of ω-conotoxin GVIA, and further indicates that the spontaneous release of acetylcholine is not dependent on calcium influx through the N-type calcium channels.

Substitution of calcium with magnesium in the buffer was completely without effect on the contractions induced by carbachol and the carbamate physostigmine. This was unexpected, since the calcium antagonists produced a rightward shift in the dose response relationship to carbachol. It may, however, be due to incomplete removal of extracellular calcium caused by leakage of calcium from the tissue to the extracellular compartment. The soman induced contractions were, on the other hand, greatly reduced after removal of extracellular calcium. Physostigmine and soman are presumed to induce contractions by the same mechanisms, i.e. accumulation of spontaneously released acetylcholine, and the time-course and shape of the contractions were very similar. Previously it has been shown that the prejunctional effect of soman on the nerve does not affect spontaneous release of acetylcholine (Aas *et al.* 1987). The reduction of soman but not physostigmine induced contractions in calcium-free buffer, therefore indicates that soman, in addition to acetylcholinesterase inhibition, may have a direct postjunctional effect on the smooth muscle. A possible mechanism for the direct postjunctional effect of soman on the smooth muscle could be phosphorylation of proteins that are involved in the recruitment of calcium.

In conclusion, the results of this study show that the calcium antagonist ω-conotoxin GVIA potently inhibits the electrically induced calcium-dependent release of acetylcholine also in airway smooth muscle. ω-Conotoxin GVIA does not have any direct effect on the airway smooth muscle contraction and therefore has no effect on the soman induced contraction. The organic calcium antagonists, on the other hand, seem to exert their primary effect postjunctionally. Gallopamil was most potent, followed by verapamil and diltiazem. The organic calcium antagonists are effective against soman induced bronchoconstriction only in concentrations that induce severe systemic adverse effects. Their potential as therapeutic agents in treatment of organophosphate intoxication may therefore be limited to topical application.

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Table 1. The effect of calcium channel blockers on bronchial smooth muscle contraction induced by electrical field stimulation or soman.

Calcium antagonist	IC_{50} (M)					
	Electrical field stimulation			Soman ($1.4x10^{-7}$ M)		
ω-Conotoxin GVIA	$4.1x10^{-8}$	*	(4)	---	-	(6)
Verapamil	$1.6x10^{-5}$	ns	(9)	$3.5x10^{-6}$	ns	(6)
Diltiazem	$2.3x10^{-5}$	*	(6)	$2.5x10^{-5}$	*	(6)
Gallopamil	$9.7x10^{-6}$		(6)	$2.9x10^{-6}$		(6)

The results are expressed as the IC_{50} values, i.e. the concentration of calcium antagonists that inhibit the induced contraction by 50 percent. Geometric means (number of experiments in parenthesis) and significance of difference from gallopamil are shown. --- indicates only a minor effect of ω-conotoxin GVIA on the contraction induced by soman.

Electrical field stimulation: * : $p<0.017$; ns : $p\geq0.017$

Soman: * : $p<0.025$; ns : $p\geq0.025$

Table 2. The effect of calcium channel blockers on carbachol induced bronchial smooth muscle contraction.

Calcium antagonist	EC_{50} (M)	α	n
Control	$5.1x10^{-7}$	1.01±0.12	27
ω-Conotoxin GVIA	$4.1x10^{-7}$ ns	0.73±0.14 ns	4
Verapamil	$2.5x10^{-6}$ ***	1.00±0.16 ns	9
Gallopamil	$3.7x10^{-6}$ ***	0.85±0.18 ns	6
Diltiazem	$2.3x10^{-6}$ **	1.08±0.23 ns	6

The apparent affinity (EC_{50}) and the intrinsic activity (α) of carbachol in the presence of calcium antagonists are expressed relative to a control stimulation. A maximal contraction to carbachol was obtained at $1.0x10^{-4}$M. The concentrations of the calcium antagonists were: ω-conotoxin GVIA, $3.0x10^{-6}$ M; verapamil, gallopamil, and diltiazem, $1.4x10^{-4}$ M. Geometric mean (EC_{50}), mean ± SEM (α), significance of difference from individual controls (geometric mean and mean ± SEM of all controls are shown) and number of experiments (n) are shown. *** : $P<0.001$; ** : $P<0.01$; ns : $P\geq0.05$.

Table 3. The effect of calcium channel blockers and calcium-free buffer on sub-maximal bronchial smooth muscle contractions induced by carbachol, soman or physostigmine.

Drug	Control (contraction relative to maximal by Carbachol (α=1.00))	Contraction response relative to control (=1.00) in the presence of calcium antagonists or in calcium free buffer.				
		ω-Conotoxin GVIA	Verapamil	Gallopamil	Diltiazem	Calcium-free buffer
Carbachol ($1.0x10^{-6}$M)	0.60±0.02 (n=27)	0.99±0.04 (n=8) ns	0.36±0.04 (n=7) **	0.07±0.02 (n=6) ***	0.45±0.05 (n=6) *	0.93±0.13 (n=5) ns
Soman ($1.4x10^{-7}$M)	0.52±0.05 (n=33)	0.85±0.03 (n=6) ns	0 (n=6) ***	0 (n=6)***	0.07±0.02 (n=6) ***	0.08±0.04 (n=5) *
Physostigmine ($5.0x10^{-6}$M)	0.51±0.5 (n=4)	-	-	-	-	0.86±0.15 (n=5) ns

The effects of calcium antagonists or calcium-free buffer on sub-maximal contractions (50-60%) induced by carbachol, soman and physostigmine (only calcium-free buffer), are expressed relative to control (control contraction = 1.00). Calcium antagonists were added or substitution with a calcium-free buffer were done when a maximal contraction to the drugs was obtained. The concentrations of the calcium antagonists were: ω-conotoxin, $3.0x10^{-6}$M; and verapamil, gallopamil and diltiazem, $1.4x10^{-4}$M. Mean ± SEM, significance of difference from individual controls (mean ± SEM of all controls is shown) and number of experiments (n) are shown. (-) indicates that experiments were not performed.
*** : P<0.001; ** : P<0.01; * : P<0.05; ns : P≥0.05

Table 4. The effect of soman on acetylcholinesterase and butyrylcholinesterase activities in bronchial smooth muscle.

Soman (M)	Acetylcholinesterase (%)	Butyrylcholinesterase (%)	n
0	100	100	40
1.4×10^{-7}	5.1±0.3	2.2±0.2	47

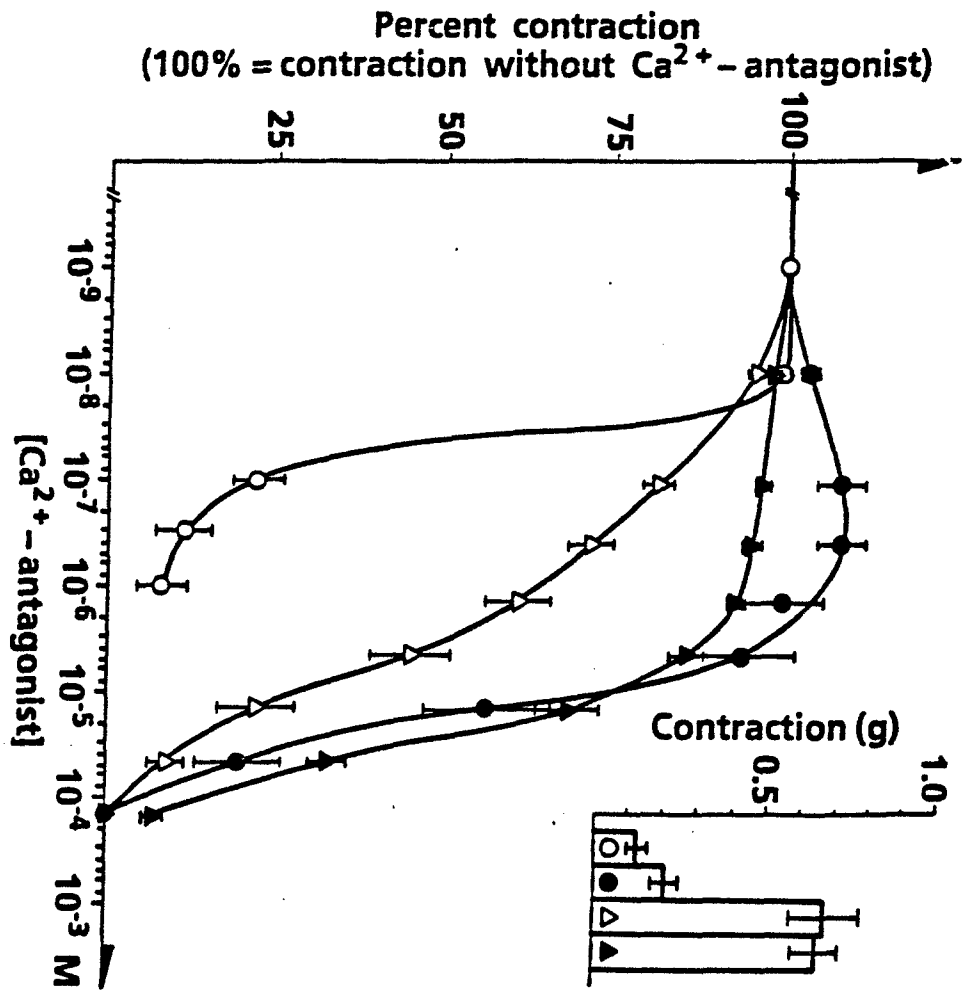
Acetylcholinesterase and butyrylcholinesterase activities in rat bronchi after treatment with soman *in vitro*. The bronchi were exposed to soman for approximately 30 min before recording the alterations in smooth muscle contraction following application of calcium antagonists. Acetylcholinesterase activity: 100%=6.3±0.22 nmol acetylcholine / min / mg protein; butyrylcholinesterase activity: 100%=9.82±0.34 nmol acetylcholine / min / mg protein. The results are expressed as mean ± SEM.

LEGENDS

Figure 1. Concentration-response curves for ω-conotoxin GVIA (O), verapamil (●), gallopamil (Δ), and diltiazem (▲) on bronchial smooth muscle contractions induced by electrical field stimulation (30 Hz and 1 ms for 3 s at 40 s intervals with supramaximal voltage). Control responses to electrical stimulation in absolute units (g) represented by columns are shown (insert upper right corner). Results are expressed as the mean in percent of control (100 percent) of 4 (O), 9 (●), and 6 (Δ and ▲) experiments, and vertical lines indicate SEM.

Figure 2. Concentration-response curves for ω-conotoxin GVIA (O), verapamil (●), gallopamil (Δ), and diltiazem (▲) on bronchial smooth muscle contractions induced by soman. The calcium antagonists were applied on maximal contraction induced by soman (approximately 10 min after application). Control responses to soman in absolute units (g) represented by columns are shown (insert upper right corner). Results are expressed as the mean in percent of control (100 percent) of 6 experiments in each group, and vertical lines indicate SEM.

Figure 1


Percent contraction
(100% = contraction without Ca2+ – antagonist)
25
50
75
100
10-9
10-8
10-7
10-6
10-5
10-4
10-3 M
[Ca2+ – antagonist]
Contraction (g)
0.5
1.0

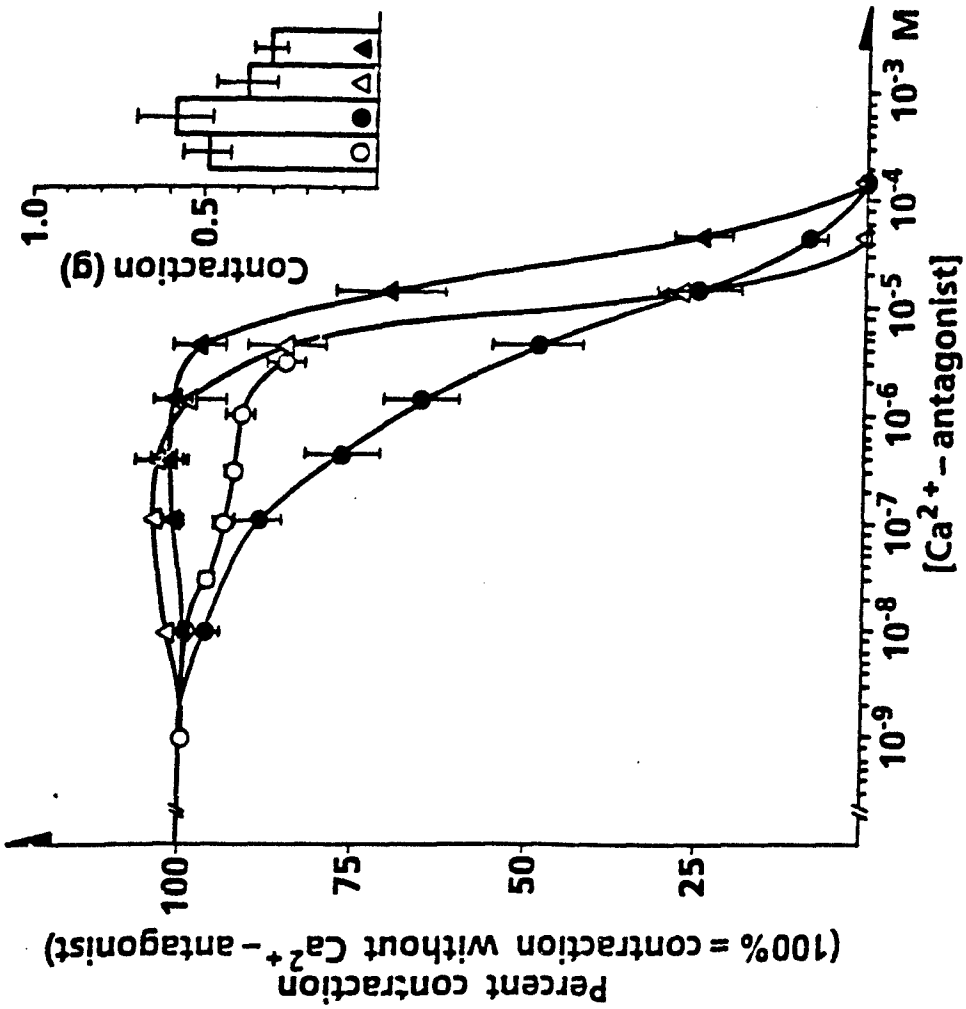

Contraction (g)
1.0
0.5
Percent contraction
(100% = contraction without Ca2+ – antagonist)
100
75
50
25
10-9 10-8 10-7 10-6 10-5 10-4 10-3 M
[Ca2+ – antagonist]

Figure 2

Paper IV

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Inhibition of serine esterases in different rat tissues following inhalation of soman

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The acute toxic effects of organophosphates (OPs) are due to inhibition of acetylcholinesterase (AChE) (EC 3.1.1.7). The inhibition of other serine esterases, such as butyrylcholinesterase (BuChE) (EC 3.1.1.8) and carboxylesterase (CarbE) (EC 3.1.1.1) does not induce any known physiological alterations. Recently, BuChE has been shown to coregulate acetylcholine lifetime in canine trachealis muscle and may therefore in some tissues play a role in the breakdown of acetylcholine (ACh) [1]. BuChE and especially CarbE may, however, be important for detoxification of low doses of OPs in rodents, since they have a high plasma concentration of CarbE [2–4].

A good correlation between the concentration of plasma CarbE and the LD_{50} of the OP soman in the developing rat has previously been shown [5]. Furthermore, injection of partially purified rat liver CarbE into 14-day-old rats increased the tolerance to soman, indicating that CarbE in plasma may be of great importance for the detoxification of organophosphorus compounds. CarbE may thereby function as a very important barrier which limits the distribution of the toxic agent to vital organs [6], since the difference in plasma concentration of CarbE between different species correlates with the difference in LD_{50} [7].

The aim of the present work was to elucidate whether CarbE in respiratory tissue and plasma plays an important role in detoxification of soman during inhalation exposure.

Materials and Methods

Chemicals. [1-^{14}C]Acetylcholine chloride ([^{14}C]ACh) was purchased from Amersham International (Bucks, U.K.). Ethopropazine (10-[2-(diethylamino) propyl]-phenothiazine) and 4-nitrophenyl butyrate were from the Sigma Chemical Co. (Poole, U.K.). Soman (*O*-[1,2,2-trimethyl-propyl]-methyl-phosphonofluoridate), assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in our laboratory. All other chemicals were of analytical laboratory reagent grade.

Inhalation method. Whole body exposures of male Wistar rats (200–300 g) (Møllegard, Copenhagen) to sub-acute concentrations of the acetylcholinesterase inhibitor soman were carried out in a dynamic inhalation system designed specifically for the exposure of small rodents to highly toxic gases [8]. Two rats were exposed simultaneously in a glass chamber of 2200 mL, the atmospheric concentration of soman was measured by gas chromatography (Carlo Erba, HRGC 5160) with a nitrogen/phosphorus detector.

No symptoms of poisoning were observed during the inhalation period.

Enzyme activity assays. The total cholinesterase (ChE) activity was determined by the radiochemical method of Sterri and Fonnum [9] at 30°. The AChE activity was measured after inhibition of BuChE by 0.2 mM ethopropazine [10]. The CarbE activity was measured by the spectrophotometric method of Ljungquist and Augustinsson [11] with modifications [12]. The protein concentration was determined by the method of Lowry *et al.* [13].

Means and standard error of the mean (SEM) were calculated for all data. The Student's *t*-test was used to assess the significance of the differences between data groups.

Results and Discussion

The airways and lungs are the first tissues exposed to toxic gases and vapours and are also the primary uptake sites for some OPs. The results from this study show that long-term exposure to low concentrations of soman primarily inhibits the cholinesterases of the respiratory tissue, plasma and erythrocytes and the CarbE of plasma and airways (Table 1). Although there were no symptoms

Table 1. Activity of AChE, BuChE and CarbE in different tissues in the rat following 40 hr inhalation exposure to two different concentrations of the organophosphorus anticholinesterase soman

Enzyme	Ct (soman) ± SEM (N = 6) (mg min/m³)	Mean per cent enzyme activity ± SEM					
		Airways	Lung	Diaphragm	Brain	Plasma	Erythrocyte
AChE	0	100 ± 9 (8.82) N = 14	100 ± 8 (3.17) N = 14	100 ± 13 (3.83) N = 14	100 ± 11 (49.2) N = 7	—	100 ± 10 (1.95) N = 12
	128 ± 25	15 ± 3 *** N = 12	17 ± 3 *** N = 12	95 ± 14 NS N = 12	123 ± 17 NS N = 8	—	15 ± 3 *** N = 12
	560 ± 77	2 ± 1 *** N = 9	5 ± 2 *** N = 9	26 ± 7 *** N = 9	59 ± 12 * N = 7	—	8 ± 3 *** N = 7
BuChE	0	100 ± 11 (17.5) N = 14	100 ± 8 (4.78) N = 14	100 ± 17 (1.55) N = 14	100 ± 22 (8.45) N = 7	100 ± 15 (1.34) N = 14	—
	128 ± 25	7 ± 3 *** N = 12	37 ± 5 *** N = 12	31 ± 12 ** N = 12	50 ± 18 NS N = 8	31 ± 9 *** N = 12	—
	560 ± 77	0 *** N = 9	8 ± 3 *** N = 9	41 ± 11 * N = 9	65 ± 23 NS N = 7	8 ± 3 *** N = 9	—
CarbE	0	100 ± 15 (280) N = 14	100 ± 10 (431) N = 14	100 ± 22 (102) N = 14	100 ± 9 (60.2) N = 7	100 ± 8 (81.8) N = 14	—
	128 ± 25	53 ± 12 * N = 12	63 ± 5 ** N = 12	81 ± 12 NS N = 12	97 ± 9 NS N = 8	39 ± 6 *** N = 12	—
	560 ± 77	25 ± 5 *** N = 9	69 ± 8 * N = 9	59 ± 12 NS N = 9	102 ± 7 NS N = 7	12 ± 2 *** N = 9	—

Activities of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and carboxylesterase (CarbE) in per cent of control (unexposed rats) after exposure to 0.05 and 0.2 mg/m³ (40 hr) of soman. Specific activity (nmol/min/mg protein) is given in parentheses for each control. The enzyme activities were determined as described in Materials and Methods. Values represent mean ± SEM of N animals.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS, $P \geq 0.05$.

of poisoning observed during the inhalation experiments, also the cholinesterases in the diaphragm were inhibited to a large extent by soman.

The inhalation experiments (40 hr) with two concentrations of soman (128 ± 12 and 560 ± 77 mg min/m³) inhibited the AChE activity in the airways (85 and 98%, respectively), lung (83 and 95%, respectively) and erythrocytes (85 and 92%, respectively) to approximately the same extent. The BuChE activity in lung (63 and 92%, respectively) and plasma (69 and 92%, respectively) were inhibited to approximately the same extent after the soman exposure, although BuChE was apparently less inhibited than AChE. Inhibition of AChE and BuChE were less pronounced in the diaphragm and the brain at both concentrations of soman than in the other tissues examined. This difference in the degree of inhibition is probably due to detoxification by covalent binding of soman to the active site of CarbE and BuChE before soman actually reaches these target tissues [2, 3, 4, 14, 15]. Furthermore, hydrolysis of soman in the liver by phosphorylphosphatases limits its accumulation in the blood [16].

The CarbE activities in diaphragm and brain were not significantly inhibited by soman. The CarbE activity in plasma, however, was significantly ($P < 0.001$) inhibited (61 and 88%, respectively) following exposure to both the high and the low concentration of soman as were the CarbE activities in airways (47 and 75%, respectively) and lung (37 and 31%, respectively). There are two main groups of CarbE in plasma that can be separated on the basis of their specificity to methyl butyrate and 4-nitrophenyl butyrate [12]. It has been suggested that the plasma CarbE with the highest specificity for 4-nitrophenyl butyrate is the most important enzyme for detoxification after injection of soman [5, 6, 12].

Our results show that inhaled soman inhibits plasma CarbE significantly more than the CarbE in lung and airways. Separate studies have shown that there is only a small difference in the bimolecular inhibition constants of plasma and lung CarbE with soman (R. Gaustad, NDRE, personal communication). Since the cholinesterases of the respiratory tissues, including lung, and the blood were equally inhibited, the small reduction of the CarbE activity in lung compared to plasma was surprising. One possible explanation is the cellular localization of the enzymes. It may be that lung CarbE is not readily available to soman. A similar difference in the level of inhibition between ChE and CarbE is seen in rat liver after *in vitro* perfusion with soman [17].

In summary, the potent inhibition of CarbE activity in plasma during long-term (40 hr) sub-acute inhalation exposure to soman indicates that CarbE in plasma represents a very important barrier to sub-acute concentrations of inhaled soman. The CarbEs in the airways and the lung are inhibited to a lower extent and thus seems not to be as important as plasma CarbE. The CarbEs in the respiratory tissue are, however, more inhibited after inhalation exposure compared to after injection of soman [12], and may accordingly play a more important role in the detoxification of inhaled soman.

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Paper V

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PULMONARY PHARMACOLOGY

Prejunctional Stimulation of Cholinergic Nerves in Rat Airway Smooth Muscle by an Adenosine Analogue

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SUMMARY. **The effect of 5'-N-ethylcarboxamidoadenosine (NECA) on rat bronchial smooth muscle was examined in vitro. Both the nerve mediated muscle contraction induced by electrical stimulation and the potassium evoked release of [^{3}H]ACh were enhanced by NECA. The apparent affinity (EC_{50}) of NECA in the contraction experiments was 0.30 ± 0.06 μM. The adenosine (ADO) receptor antagonist, 8-phenyltheophylline (8-PT), inhibited the NECA induced potentiation of both the electrical induced contraction and the potassium evoked release of [^{3}H]ACh. The EC_{50} and intrinsic activity of exogenous ACh were not altered in the presence of NECA (1 μM) in experiments where smooth muscle contraction were measured, indicating that NECA has a prejunctional effect and not a postjunctional effect on muscarinic receptors. The new A_2 specific ADO receptor agonist 2-p-(2-carboxyethyl)-phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) and ADO also enhanced the nerve-mediated contraction ($EC_{50} = 35 \pm 8$ μM and 69 ± 20 μM, respectively). 8-PT (10 μM) and enprofylline (ENPF) (10 μM) inhibited the electrically induced contraction by $55 \pm 16\%$ and $45 \pm 5\%$ respectively. The potassium evoked release, however, was stimulated $56 \pm 6\%$ and $39 \pm 7\%$ by 50 μM 8-PT and ENPF respectively. The results provide evidence for a NECA specific ADO receptor in rat bronchi that is most likely prejunctional. Stimulation of this receptor, which may be of an A_2 receptor subtype, enhances the nerve mediated release of ACh and thereby induce contraction of the bronchial smooth muscle.**

INTRODUCTION

Adenosine (ADO) is a neuromodulator both in the central and peripheral nervous system. The neuromodulatory function has until now mainly been shown to be a presynaptic inhibition of the neurotransmitter release.[1–4] There are at least two ADO receptor subtypes, A_1 and A_2, which can be differentiated on the basis of their affinity to different ADO agonists.[5,6] The A_1 receptor preferentially binds N^6-substituted ADO agonists, like R-N^6-phenylisopropyladenosine (R-PIA), while 5'-N-ethylcarboxamidoadenosine (NECA) is more potent than R-PIA at the A_2 receptor. A novel ADO analogue 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680), that is as potent as NECA on A_2 receptors and 140-fold selective for A_2 compared to A_1 in radioligand binding experiments in rat brain, has recently been developed.[7] The neurotransmitter release inhibiting effect of ADO is assumed to be mediated through stimulation of presynaptic A_1 ADO receptors, but the precise mechanism by which the release is inhibited is not yet clear.[8] On the other hand, a few data have been published, indicating that selective A_2 ADO receptor activation and augmented cAMP levels can enhance neurotransmitter release in brain slices.[9–11]

Methylxanthines are known to relax airway smooth muscle, but whether this effect is mainly due to inhibition of intracellular cyclic nucleotide phosphodiesterase or extracellular ADO receptor antagonism or some other unknown mechanism is still a matter of controversy. Methylxanthines relax airway smooth muscle at concentrations lower than normally required for cyclic nucleotide phosphodiesterase inhibition.[12–14] On the other hand, the relaxation of airway smooth muscle by methylxanthines via ADO receptor antagonism, would require a muscle tonus induced by ADO, which is usually considered as a smooth muscle relaxant. It has been shown, however, that ADO causes airway contraction exclusively in asthmatic human subjects,[15] and some reports have shown a dual effect of ADO on airway tissue, i.e. relaxation of precontracted tissue and contraction of resting tissue.[16,17] Furthermore, an enhancement of the cholinergic nerve-induced contraction by ADO A_2 receptor stimulation with NECA, has been shown in rabbit bronchi.[18] However, the mechanism behind this effect is unknown at present. Antagonism of the enhancing effects of ADO on airway contraction may contribute to the airway relaxing effect induced by methylxanthines in the therapeutic treatment of obstructive lung disease.

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In the present experiments we have examined the effect of NECA, CGS 21680, ADO, 8-phenyltheophylline (8-PT) and enprofylline (ENPF) on the cholinergic neurotransmission in rat bronchial smooth muscle.

METHODS

Chemicals

[^{3}H](methyl)-choline chloride was purchased from New England Nuclear (Boston, MA, USA). CGS 21680 was kindly provided by Dr Lovell, Ciba-Geigy (Summit, NJ, USA) and enprofylline (ENPF) was kindly provided by Draco (Lund, Sweden). Hemicholinium-3 was bought from Aldrich (Steinheim, FRG), acetylcholine (ACh) from Fluka (Buchs, Switzerland) and carbachol from BDH (Poole, UK). The following chemicals were obtained from Sigma (Poole, UK): ADO, NECA, R-PIA, dipyridamole, 8-phenyltheophylline (8-PT) and adenosine deaminase (ADA) (from calf intestine). All other chemicals were of analytical laboratory reagent grade.

Animals

Male Wistar rats (200–300 g) (Møllegard, Copenhagen) were kept in standard laboratory cages for 1–2 weeks with free access to standard laboratory diet and water. The light/dark cycle was 12 h, the relative humidity 45–55% and the temperature 22–25°C. The animals were kept on sawdust which was replaced daily.

Physiological method

The rats were killed by decapitation and the bronchi were removed and transferred to the physiological buffer. The left and right bronchi were mounted as circular preparations on hooks made from cannulas and the contractions were measured as diameter reductions as previously described by Aas and Helle.[19] The thermostatically controlled (37°C) organ-bath contained 6 ml Krebs buffer of the following composition (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; glucose, 11.1. The solution was gassed with 95% O_2 + 5% CO_2 (pH 7.4). A preload of 1.0 g was applied and the preparations were equilibrated for at least 30 min before the start of the experiments. Platinum electrodes connected to a Grass S88 Stimulator were used for electrical stimulation. The stimulation parameters were 20 Hz and 1 ms for 3 s at 40 s intervals at supramaximal voltage. The transmurally induced contractions before addition of drugs were used as control in each experiment, and the response in the presence of drugs was calculated as percent increase or decrease relative to control (control = 100%). The contractions were recorded isometrically by Grass Force Displacement Transducers (FT03C) and monitored on a Grass Polygraph (model 79E) fitted with amplifiers (7 P 1A).

Release of [^{3}H]ACh

Both primary bronchi were dissected out, opened ventrally, and cut into small pieces (approximately 1 mg wet wt) as previously described by Aas and Fonnum.[20] The tissue was preloaded with [^{3}H]choline by incubation at 25°C for 60 min in buffer A (see below) containing 1.1 μM [^{3}H]choline chloride (10 Ci mmol^{-1}), in a shaking water bath. After incubation, the tissue was washed twice in buffer A, and superfused for 60 min before collection of samples. The superfusion chambers were made of two disposable pipette tips which were cut in half transversely and joined together. A flow rate of 200 μl min^{-1} was generated by a peristaltic pump (Ole Dichs, 6 channels). The media were kept in a thermostatically controlled water bath at 25°C during the experiments. The superfusion buffer (buffer A) had the following composition (in mM): NaCl 140.0, KCl 5.1, $CaCl_2$ 2.0, $MgSO_4$ 1.0, Na_2HPO_4 1.2, Tris-HCl 15.0, glucose 5.0. The depolarization buffer was as buffer A, but contained 51 mM KCl, and the concentration of NaCl was reduced accordingly to keep the ionic strength constant. In addition, all the superfusion media contained hemicholinium-3 (10 μM) to inhibit the high affinity uptake of choline.[21] Three periods with potassium stimulation (51 mM) (S1, S2 and S3) for 5 min were separated by 35 min superfusion with buffer A. Drugs were always added 5 min before and were also present during the second potassium stimulation (S2). Each experiment with drugs had its own control experiment. The collected fractions of the superfusion media were counted in an OPTI-FLUOR scintillation cocktail for aqueous and nonaqueous samples.

Data analysis

The fractional rate of evoked release of [^{3}H]ACh was calculated using a computer. Release of tritium during stimulation of tissues preincubated with [^{3}H]choline has been shown to be a good measure for [^{3}H]ACh release.[22] We have therefore used the term [^{3}H]ACh release when evoked tritium release were measured. Peak areas, as well as basal release before and after the depolarization period and ratios between peak areas, were calculated. [^{3}H]ACh release was calculated by subtracting the basal release from the evoked release ([^{3}H]ACh release/basal tritium release = 0.46 ± 0.03 (from 12 randomly chosen control experiments)). Potassium evoked release was always performed three times consecutively, separated by 35 min superfusion with buffer A. The potassium evoked

release of [^{3}H]ACh was calculated as a percent of that released in the first stimulation in each experiment. Mean and standard error of the mean (SEM) were calculated for all data. Significance for differences were calculated by Student's t-test.

RESULTS

Physiological experiments

The transmural nerve stimulation, which induced release of ACh and subsequent contraction of the primary bronchial smooth muscle, as previously shown by Aas and Helle,[19] were enhanced in a concentration dependent manner by NECA (Figs 1B and 2), CGS 21680 and ADO (Fig. 2). The apparent affinities (EC_{50}) of NECA, CGS 21680 and ADO were calculated from the sigmoidal dose response curves and were 0.30 ± 0.06 μM, 35 ± 8 μM and 69 ± 20 μM, respectively (Fig. 2). To prevent the uptake of ADO, dipyridamole (2 μM) was added 6 min before the addition of ADO. Neither NECA, CGS 21680 nor ADO had any effect on the baseline tension. NECA (1 μM) affected neither the intrinsic activity nor the EC_{50} of the exogenously applied ACh (Fig. 3).

When 10 μM 8-PT or ENPF was added to the organ-bath, the nerve evoked contractions were reduced by $55 \pm 16\%$ (n = 11) and $45 \pm 5\%$ (n = 6), respectively, but the baseline tension was not affected (Fig. 1C, D). Addition of ADA (4 U/ml) 10 min before exposure to 8-PT and ENPF changed the reduction slightly to $37 \pm 4\%$ and $25 \pm 6\%$ (n = 6) respectively. In the presence of 10 μM ENPF, there was no significant change in EC_{50} of NECA (EC_{50} = 0.36 ± 0.10 μM). On the other hand, 8-PT (10 μM) shifted the dose response curve of NECA significantly to the right, and thereby increased the EC_{50} for NECA to 4.5 ± 1.3 μM (Fig. 2). The maximal enhancement of the electrically induced contractions produced by NECA, CGS 21680 and ADO was $47 \pm 9\%$ (n = 10), $72 \pm 23\%$ (n = 10) and $47 \pm 10\%$ (n = 6), respectively.

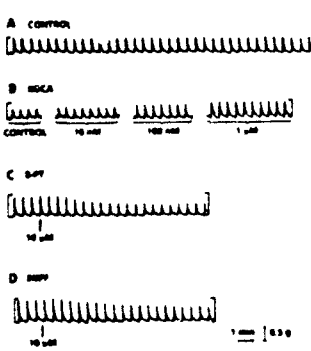

Fig. 1—Tracings showing muscle contractions elicited by electrical field stimulation: (A) control, (B) in the presence of different concentrations of NECA, (C) the effect of 10 μM 8-PT, and (D) the effect of 10 μM ENPF on electrical induced contractions.

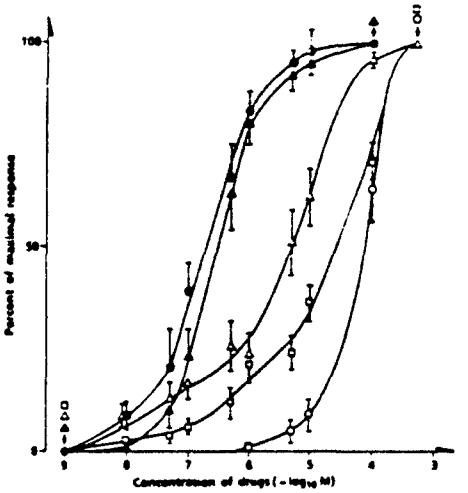

Fig. 2—Dose-response curves for stimulation of electrically evoked contractions of rat bronchi: ADO in the presence of 2 μM dipyridamole (○) (0% = 0.32 g, 100% = 0.56 g); CGS 21680 (□) (0% = 0.26 g, 100% = 0.39 g); NECA (●) (0% = 0.46 g, 100% = 0.65 g); NECA in the presence of 10 μM 8-PT (△) (0% = 0.32 g, 100% = 0.54 g); NECA in the presence of 10 μM ENPF (▲) (0% = 0.25 g, 100% = 0.53 g). ADO, CGS 21680 and NECA potentiated the transmurally stimulated nerve-mediated mechanical response (1 ms, 20 Hz). The responses are the means ± SEM of 6–11 experiments and are plotted as percent of maximal response for each experiment.

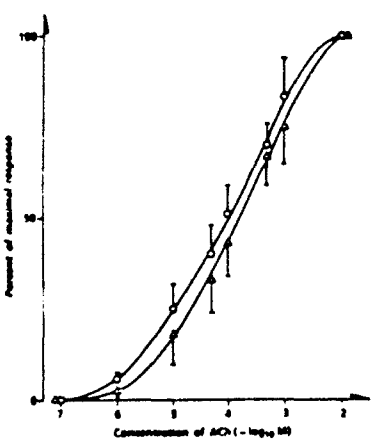

Fig. 3—Dose-response curves for bronchi contractions elicited by exogenously applied ACh: control (○) (100% = 0.84 ± 0.09 g, n = 12); in the presence of 1 μM NECA (△) (100% = 0.91 ± 0.10 g, n = 6). The values represent mean ± SEM.

The presence of ADA (4 U/ml) in the organ bath did not change the electrically induced contractions

or the baseline tension significantly. Furthermore, there were no observed effects on the transmurally induced contractions or the baseline tension when R-PIA was applied in the same concentrations as NECA (1 nM–0.5 mM, not shown).

Release experiments

Potassium evoked release of [^{3}H]ACh from rat primary bronchi preloaded with [^{3}H]choline was examined in the presence of NECA and CGS 21680, in order to confirm that the stimulation of the cholinergic contractile response by these drugs was prejunctional (Table 1). It has previously been shown that the potassium evoked release of [^{3}H]ACh from rat primary bronchi is Ca^{2+}-dependent.[20] Figure 4 shows the potassium (51 mM) evoked release of [^{3}H]ACh in bronchial smooth muscle tissue from rat. The increase in transmitter release was rapid and it decreased to the basal release level after reexposure to the low potassium buffer (buffer A). Repetitive stimulations produced only a minor overall decrease in the release of [^{3}H]ACh in the presence of hemicholinium-3 (Fig. 4).

NECA in concentrations of 50 nM and 5 μM, stimulated the second potassium evoked release (S2) by 31% and 54% respectively. The stimulation was of long duration, i.e. the third potassium evoked release (S3) 35 minutes after the NECA exposure was, compared with S3 in the control experiment, still enhanced, with 61% and 73% respectively. CGS 21680 or R-PIA in the concentration range 100 nM–500 μM did not affect the potassium stimulated release of [^{3}H]ACh (Table 1).

8-PT (50 μM) and ENPF (50 μM) stimulated the

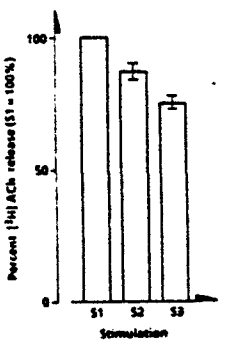

Fig. 4—The release of [^{3}H]ACh induced by stimulation of pieces of bronchial smooth muscle with 51 mM potassium for 5 min in the presence of 2 mM calcium. Repetitive potassium evoked release is calculated in percent of the first stimulation (S1). The three potassium evoked stimulations are separated by time intervals of 35 min and superfusion with buffer A. The columns represent mean ± SEM of 51 control experiments.

Table 1. Potassium induced [^{3}H]ACh release with different drugs present 5 min before and during the second potassium-stimulation (S2)

Experiment	Drug	Concentration	Percent release ± SEM S2	S3
A	NECA	50 nM	131 ± 4 **	161 ± 5 **
B	NECA	5 μM	154 ± 7 **	173 ± 7 **
C	CGS21680	100 nM	108 ± 5 ns	107 ± 8 ns
D	CGS21680	500 μM	92 ± 3 ns	97 ± 4 ns
E	8-PT	50 μM	156 ± 6 **	118 ± 5 ns
F	8-PT + ADA	50 μM + 4 U/ml	128 ± 8 *	103 ± 5 ns
G	NECA + 8-PT	5 μM + 5 μM	133 ± 6 *	158 ± 6 **
H	NECA + 8-PT	5 μM + 50 μM	116 ± 8 ns	123 ± 8 ns
I	ENPF	50 μM	139 ± 7 **	105 ± 7 ns
J	ENPF + ADA	50 μM + 4 U/ml	111 ± 3 *	98 ± 6 ns
K	NECA + ENPF	5 μM + 50 μM	116 ± 4 *	100 ± 6 ns
L	ADA	4 U/ml	84 ± 3 **	121 ± 6 *
M	R-PIA	50 nM	105 ± 4 ns	105 ± 4 ns
N	R-PIA	500 μM	99 ± 6 ns	148 ± 18 ns

Bronchial smooth muscle was stimulated for 5 min three times consecutively with potassium (51 mM). The drugs (NECA, CGS21680 8-PT, ENPF and ADA) were present 5 min before and during the second stimulation (S2), but not during the first (S1) or the third stimulation (S3). Two different drugs were applied simultaneously in some experiments (F, G, H, J and K). The time interval between the three consecutive stimulations were 35 min. A control experiment without drugs present during S2 was performed for each experiment, except in experiment F and J where ADA were present in the control. The potassium evoked release in S2 and S3 were calculated relatively to the first potassium evoked release in both control and drug exposed experiments. The values represent release in S2 and S3 in the drug exposure experiments with the corresponding release in the control experiments as 100%. Each value is calculated from 12 experiments (6 control + 6 exposed), and SEM is calculated for all data. **$p < 0.01$; *$p < 0.05$; ns $p \geq 0.05$.

potassium evoked [^{3}H]ACh release in S2 by 56% and 39% respectively, but did not produce any significant effect on the evoked [^{3}H]ACh release in S3 compared with control. The stimulation of [^{3}H]ACh release by 8-PT and ENPF were significantly reduced to 28% and 11% in the presence of 4 U/ml ADA. When 5 μM and 50 μM 8-PT were added together with 5 μM NECA, an inhibition of the stimulating effect of NECA on the potassium evoked release was observed. Furthermore, the release stimulating effect of 8-PT was also inhibited in the presence of NECA. When 50 μM ENPF was added together with 5 μM NECA, only 16% stimulation of the evoked release in S2 was observed.

In order to examine the role of endogenous ADO, 4 U/ml of ADA was added 5 min before and during the second potassium stimulation (S2). The presence of ADA resulted in a very small but significant decrease (16%) of the S2 stimulated release.

DISCUSSION

The present experiments provide evidence for an ADO receptor mediated enhancement of the cholinergic response in rat primary bronchial smooth muscle. The enhancement seems to be due to prejunctional stimulation of ACh release, probably by stimulation of a NECA specific A_2 related receptor.

The enhancement of the nerve evoked contractions by NECA was inhibited by 8-PT (10 μM). 8-PT is a selective ADO receptor antagonist with low phosphodiesterase inhibitory activity,[23] indicating that the effect of NECA is mediated through an ADO receptor. Addition of CGS 21680 also enhanced the nerve-mediated contractions, but not as potently as NECA. The A_1 selective ADO analogue R-PIA did not produce any significant alteration of the electrically evoked contractions in concentrations up to 500 μM. The order of potency together with the lack of effect of R-PIA is unexpected, and we cannot exclude the possibility that a novel ADO receptor with a different agonist profile than both A_1 and A_2 may be involved.

NECA did not induce enhancement of contractions elicited by exogenously applied ACh, indicating that the effect is mediated through a prejunctional mechanism and not a postjunctional receptor. This was confirmed by the release experiments where both 50 nM and 5 μM of NECA stimulated the potassium induced [^{3}H]ACh release. Furthermore, the NECA induced enhancement was inhibited by the ADO receptor antagonist 8-PT. The A_2 selective agonist CGS 21680 did not affect the release of [^{3}H]ACh, indicating that the effect of CGS 21680 may be postjunctional as previously shown with ADO.[20] The A_1 selective agonist R-PIA did not produce any significant effect on the potassium evoked release of [^{3}H]ACh. This is unexpected since R-PIA normally stimulates A_2 receptors although less potent than NECA, but in agreement with the results from the contraction experiments.

It has previously been shown that ADO is unable to affect cholinergic neurotransmission in the guinea pig trachea.[24] On the other hand, enhancement of cholinergic neurotransmission by ADO, probably via an A_2 receptor, has previously been shown in rabbit bronchi, but the mechanism behind this effect is not clear.[18] The same effect is evidently present in the rat primary bronchi, as presently shown, where NECA stimulates the [^{3}H]ACh release. Enhancement of evoked transmitter release has previously been shown in brain slices by stimulation with NECA, and also in cultured cerebellar cells by stimulation with R-PIA after pertussis toxin treatment,[10,11] but has until now not been shown in any peripheral tissues.

ENPF is an anti-asthmatic xanthine drug which has been reported to be without ADO antagonistic properties.[25,26] However, recent evidence has been presented which indicates that ENPF may exhibit ADO receptor antagonistic properties at A_1 receptors on rat fat cells and at A_2 receptors on human platelets.[27] ENPF (10 μM) did not alter the apparent affinity, EC_{50}, of NECA in our experiments where nerve-mediated contractions of rat bronchi were enhanced. This is in agreement with the reported lack of antagonistic properties of ENPF on ADO receptors. ENPF may however exhibit some antagonistic properties, since a higher concentration (50 μM) significantly reduced the NECA induced stimulation of the potassium induced [^{3}H]ACh release.

Both 8-PT and ENPF produced an inhibition of the nerve evoked contraction at a concentration (10 μM) which is lower than what is normally required for the inhibition of the cyclic nucleotide phosphodiesterase activity. A part of the inhibition of contraction is, however, probably due to ADO receptor antagonism, since the presence of ADA reduced the inhibition slightly, and exogenously applied ADO enhanced the electrically stimulated contractions. The inhibitory effect of 8-PT and ENPF was, however, probably not due to antagonism of the presynaptic NECA stimulated ADO receptor mediating enhancement of release, since both 8-PT and ENPF stimulated the potassium evoked release of [^{3}H]ACh. A stimulatory effect on release by alkylxanthines has earlier been observed in brain slices from rat, where both caffeine and aminophylline altered the electrically induced ACh release in a concentration dependent biphasic manner.[28] It has been suggested that this action was due to displacement of endogenous ADO from ADO receptors mediating inhibition of release. In agreement, in the present experiments the effect of 8-PT and ENPF were significantly reduced in the presence of ADA, but the release was still enhanced by the alkylxanthines compared to control. These results indicate that the stimulation may be

due to both ADO receptor antagonism and some other effect of the alkylxanthines.

In conclusion, the present results provide evidence for a stimulating effect of NECA on the cholinergic induced contraction of rat bronchial smooth muscle. The stimulation by NECA seems to be mediated predominantly by a prejunctional NECA specific ADO receptor on cholinergic nerves which enhance nerve evoked release of ACh in rat bronchial smooth muscle. The receptor is most likely related to the A_2 receptor subtype.

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